# ONTOGENY OF OSMOREGULATION OF THE EMBRYOS OF TWO INTERTIDAL CRABS *HEMIGRAPSUS EDWARDSII* AND *HEMIGRAPSUS CRENULATUS*

---

A thesis

submitted in fulfilment of

the requirements for the Degree

of

Doctor of Philosophy in Zoology

in the

University of Canterbury

by

Deepani Seneviratna

---

**University of Canterbury**

**2003**

# CONTENTS

## ABSTRACT

The effects of variations in salinity on the developing embryos of two intertidal crabs, *Hemigrapsus edwardsii* and *Hemigrapsus crenulatus* were studied in relation to short term (6 – 96 h) and long term (continuous) exposure to low salinities from spawning to hatching.

For the purpose of describing physiological and morphological changes during development, developing embryos of both species were grouped into 5 stages that were timed under standard conditions in the laboratory; Stage (1) Cleavage to Blastula, (2) Gastrula, (3) Eyespot & chromatophores, (4) Yolk in 4 lobes and (5) Yolk in 2 lobes. The total incubation periods of embryos from spawning to hatching of *H. edwardsii* and *H. crenulatus* were 62.0 ± 3.1 and 43.0 ± 1.8 days respectively at a constant temperature of 15 °C. Embryos of *H. edwardsii* (20 nL stage 1 to 35 nL stage 5) are larger than the embryos of *H. crenulatus* (7 nL to 18 nL). Ultrastructural studies revealed the presence of two distinct egg membranes in these embryos. Silver staining demonstrated a distinct patch on the surface of the embryos of both species, presumably corresponding to an area of high chloride permeability. This structure appeared at the gastrula stage and was present throughout the embryonic development until hatching when it disappeared. It is suggested that this structure corresponds to the "embryonic dorsal organ" and plays a role in either the uptake or excretion of salts.

Salinity tolerance experiments revealed that the postgastrula stage embryos of both species are remarkably tolerant of periods of dilution, even down to 1% seawater, for many hours and that they hyperosmoregulate throughout development. Pregastrula embryos (stage 1) were nearly isosmotic in all salinities and were less tolerant of hyposaline exposure. Normal development and successful hatching occurred in 50% seawater for both species provided the exposure commenced after gastrulation. Thus, the gastrula stage appears to be the critical stage when the capacity to osmoregulate commences.

The four cations $Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$ (and associated anions) were major osmotic effectors in the embryos of *H. crenulatus* but contributed only about half of the total osmolality. $Na^+$ and $K^+$ were the main cations in these embryos. The average

concentrations of all four cations were better regulated than was total osmolality indicating the involvement of other osmolytes in osmoregulation.

$Na^+/K^+$ ATPase activity was undetectable in stage 1 embryos but rose steadily between gastrulation and hatching. A relatively larger increase in the activity of this ion transport enzyme at the Yolk in 4 lobe stage in embryos incubated in 50% seawater supports its involvement in osmoregulation.

Tritiated water and $^{22}Na$ efflux data indicated that relative to the size of the embryos, the egg membranes of *H. crenulatus* are very permeable to water and ions throughout development. There were two components of water and sodium exchange with the seawater in all embryos; a rapidly exchanging pool and a slowly exchanging pool. This rapidly exchanging pool may represent the external embryonic membranes and compartments while the slowly exchanging pool is thought to represent the embryo/yolk. Half times for turnover of embryonic (slow pool) water and sodium ranged from 0.25 to 1.25 h and 1 to 6 h respectively, throughout development. As the embryos maintain a hyperosmotic state for much longer times than these they must be actively hyperosmoregulating. That is they must be in a dynamic steady state involving the active uptake of salts and excretion of osmotically entering water.

Oxygen consumption rates of both *H. edwardsii* and *H. crenulatus* increased during development and the rate increase was greater at the end of the incubation period. Rates of oxygen consumption of embryos in acute exposure to dilute seawater varied with salinity and exposure time for all developmental stages. There was a significant increase in the total metabolic cost of development, in terms of oxygen consumed, for embryos developing in 50% seawater compared with development in normal seawater. This could be attributed primarily to an extended development time rather than an increased metabolic rate at any stage.

# CHAPTER ONE

## GENERAL INTRODUCTION

In intertidal zones and estuaries, freshwater run off, rain, and the emersion and submersion of the shore zone produce complex temporal and spatial variations of salinity. For animals inhabiting such areas, the effects of salinity change may be manifested at any stage of their life cycle. In decapod crustaceans, fertilized eggs following extrusion are clustered on pleopod setae beneath the abdomen of the female and carried for periods of days to months until they hatch. The eggs of euryhaline crabs would therefore be expected to be exposed to variations of salinity on the shore intermittently. However, since at the early stages, embryos do not possess the differentiated organs and tissues employed by adults for osmotic and ionic regulation, it is unclear how developing embryos within these eggs protect themselves from changes in the concentration of the medium.

There are several possible mechanisms of survival when externally developing embryos are confronted with salinity variations in their environment; e.g. eggs protected by envelopes, maternal crab avoids extremes at critical times, embryos tolerate but osmoconform (allow the osmotic concentration of its body fluids to correspond to that of the medium), or embryos osmoregulate (maintain or regulate its osmotic concentration in spite of external concentration changes).

The effects of environmental salinity on the internal osmolality and specific ion regulation of adult and larval crustaceans have been investigated in numerous studies (Aladin & Potts, 1995; Anger, 1996; Anger *et al.*, 2000; Brito *et al.*, 2000; Brown *et al.*, 1992; Charmantier & Charmantier-Daures, 1991; Charmantier *et al.*, 2001, 2002; Costlow *et al.*, 1966; Diesel, 1998, Guerin & Stickle, 1997; Kalber & Costlow, 1968; Lemos *et al.*, 2001; Mantel & Farmer, 1983; Pandian, 1970; Zanders, 1992). Anger (1996) reported that in larvae and first juvenile stages of *Armases miersii* extremely low and high salinities (5, 45-55 ppt) caused prolonged development durations and

increased mortality rates. His observations further suggested that some osmoregulatory capability exists from hatching. Anger and Charmantier (2000) investigated the salinity tolerance during development from hatching to the end of the first juvenile stage of *Sesarma curacaoense*. They found successful development through metamorphosis in the full salinity range tested (15 – 32 ppt).

In contrast there is relatively little information concerning the osmo-ionic regulation mechanisms of developing embryos (Bas & Spivak, 2000; Brown & Terwilliger, 1992; Charmantier, 1988; Charmantier & Charmantier-Daures, 1991, 2001; Charmantier *et al.*, 1988; Jones & Simons, 1982; Leelapiyanart, 1996; Susanto & Charmantier, 2000, 2001). Nevertheless, a few studies imply that some crab embryos are able to deal with salinity variations from an early stage. Previous research conducted at the University of Canterbury has indicated that some estuarine crabs (*Macropthalmus*, *Hemigrapsus* species) are capable of completing embryonic development in seawater diluted as much as 50% while the embryos of some other crabs (*Heterosius*, *Cyclograpsus*) survive much lower salinity for several days and hyper-regulate (Clark, 1987; Leelapiyanart, 1996). In the mud crab, *Macropthalmus hirtipes*, embryonic development was completed successfully in 18 and 36 ppt, but in 11 ppt, development did not proceed (Jones & Simons, 1982).

Studies of the ontogeny of osmotic and ionic regulation of different crustaceans revealed that the ability to osmoregulate is gained at different stages of their life cycle (Brown & Terwilliger, 1992; Charmantier & Charmantier-Daures, 2001 (review); Felder *et al.* 1986; Kalber & Costlow, 1968; Morritt & Spicer, 1995). Morritt and Spicer (1995) suggested that, in amphipods, osmoregulatory functions change with ontogeny and suggested that the vitelline membrane and the intimately associated dorsal organ act as the osmoregulatory organ. Potential sites for osmoregulation on the internal surface of the branchiostegite and adjacent body wall in the prehatched larvae of *Callinassa jamaicense* were demonstrated by Felder *et al.* (1986). Charmantier and Charmantier-Daures (1991) documented that in *Cancer irroratus* and in *C. borealis* , osmoregulation and salinity tolerance correlated and are modified at metamorphosis. In both of these species, zoeae were hyper-osmoconformers, adults were isosmotic in high salinities and slightly hyper-regulators in low salinities. In *Sesarma curacaoense,* the capacity to hyperosmoregulate in dilute media increased

gradually from hatching throughout the larval and juvenile development. In seawater (32 ppt) and at an enhanced salt concentration (44 ppt), the zoeal stages remained hyperosmoconformers (Anger & Charmantier, 2000).

Evidence for the involvement of $Na^+/K^+$ ATPase in salt uptake by adult and larval crustaceans comes from several sources (Flik & Haond, 2000; Harris & Bayliss, 1988; Lucu & Flik, 1999; Morris & Edwards, 1995; Thuet *et al.*, 1988; Towle, 1990). Localization of this enzyme at high specific activities in the epithelia of recognized sodium and chloride regulatory effector organs, notably the gills of crustaceans have been demonstrated by Lucu (1990), Lucu & Flik (1999), Mantel & Farmer (1983) and Siebers *et al.* (1985). Osmoconforming marine species show low levels of enzyme activity which do not respond to a necessarily limited range of salinity modifications. Osmoregulating estuarine species exhibit moderate levels of enzyme in posterior gills, the activity increasing as the difference in salt concentration between blood and medium increases (Towle, 1984). High specific activity in the larval salt gland of *Artemia salina*, which function as the site of water and salt uptake in these animals, was reported by Ewing & Peterson (1974). These sites correspond to sites of active ion uptake and also renal or extra-renal ion excretion. It has also been observed that there is a strong correlation between the development of osmoregulatory epithelia, the ability to osmoregulate and the activity of $Na^+/K^+$ ATPase (Bouricha *et al.*, 1994; Charmantier, 1998; Charmantier & Charmantier-Daures, 2001; Felder *et al.*, 1986).

It has frequently been postulated that the osmoprotection of externally developing embryos of decapod crustaceans is provided by the possession of relatively impermeable egg envelopes (Charmantier & Charmantier-Daures, 2001). However, several researchers have documented that throughout the embryonic growth the egg covering is permeable to water and is selectively permeable to salts and other solutes, depending on their molecule size (Charmantier & Aiken, 1987; Jensen *et al.*, 1993; Leelapiyanart, 1996; Pandian, 1970; Winnicki & Slomianko, 1970; Yonge, 1946).

Osmoregulation requires metabolic energy. There are both direct costs (osmotic work) and indirect cost ("stress", activity, etc.). Metabolic reserves (yolk) are limited in eggs. Therefore, osmoregulation may conflict with development and larval fitness. Aspects of embryonic metabolism in crustaceans have been dealt with by many

authors (Lardies & Wehrtmann, 1996; Naylor & Bennett, 1999; Taylor & Leelapiyanart, 2001; Valdes *et al.*, 1991; Wear, 1974; Wehrtmann & Kattner, 1998). An increase in the rates of oxygen consumption (as a measure of metabolic rates) with an increasing osmotic difference indicates higher energy requirements for osmotic and ionic regulation at low salinities (Aarset & Aunaas, 1990, 1990; Einarson, 1993; Rao, 1968). In postlarvae (PL 10-PL 21) of *Litopenaeus setiferus,* an inverse relationship between oxygen consumption and growth was observed and indicated that this may be due to the changes in the mechanisms involved in the osmotic adjustments (Rosas *et al.*, 1999). Therefore, it is of interest to measure relationships between metabolic rate, salinity and developmental stage.

The New Zealand "Purple rock crab", *Hemigrapsus edwardsii* Hilgendorf, 1882 (Figure 1.1) and "Hairy-handed crab" *Hemigrapsus crenulatus* H. Milne Edwards, 1837 (Figure 1.2) are two intertidal crabs (Grapsidae) found along the coastline of New Zealand. There have been many studies on the ecology and physiology of the adults of these two crab species (Bedford & Leader, 1977, 1978; Bloomfield, 1982; Hicks, 1973; Jackson, 1976; Jones, 1976; Morton & Miller, 1968; Phillips, 1968; Williams, 1969). *H. edwardsii* is found on sheltered rocky, stony or muddy shores and on stable protected boulder beaches. This species is a late autumn-winter breeder and females have only a single batch of eggs per year. Eggs are incubated for about 6 weeks (McLay, 1988). Previous studies have estimated clutch sizes of about 26,000 eggs per female (Bennett, 1964). The adults are very euryhaline (Hicks, 1973) and are good regulators (Bedford & Leader, 1977) but no information is available on salinity tolerance of its eggs. For the present study, ovigerous female *H. edwardsii* were collected from the Waipara beach, a rocky shore (Figure 1.3 A & B) which is located about 60 km away from Christchurch City Centre (43° 05' S, 172° 50' E). My field observations in this area suggest that animals living in this area should be subject to variations in salinity due to freshwater runoff, rain and desiccation interacting with tidal effects. The osmolality of the surface seawater in this study site measured during high and low tide periods was in the range of 800-1000 mOsmol/kg.

*H. crenulatus* occupies a wide variety of habitats; under stones, burrowing in sand, mud, clay or earth, in sheltered marine or estuarine habitats and has also been recorded in sheltered places often in proximity to fresh water. This species is

ovigerous from June to February. Females lay more than one batch of eggs per season. It was reported that a female of 15.6 mm carapace width carried 8968 eggs (McLay, 1988). Eggs are incubated by the female for about 8 – 12 weeks. As for *H. edwardsii*, studies done on the effects of salinity on the adult crab revealed that *H. crenulatus* is a good regulator and can withstand a salinity range from 10-160% seawater without stress for a few days (Hicks, 1973; McLay, 1988). For the present study, ovigerous female *H. crenulatus* were collected from the Avon-Heathcote estuary (43° 33' S, 172° 44' E), which is located about 16 km from the centre of Christchurch. At low tide, 80% of the estuary is tidal mudflats dissected by river channels. Salinities within the estuarine triangle are influenced daily by river and tidal flow and seasonally by rainfall and variable discharge (Figure 1.3 C). Crabs were collected from under stones at low tide and also in traps at high tide in shallow water in the channels and over the mud flats.

There are clearly many unresolved questions with respect to the survival of developing embryos of these two crab species in such habitats. For example, what are the short and long term salinity limits for survival and normal development of these embryos? What physiological mechanisms do these eggs possess in relation to osmoprotection? Do the embryos osmoregulate and at what stage does this commence? If so, what is the metabolic cost of osmoregulation in terms of oxygen consumption? What structures mediate active ion uptake and water excretion in embryonic stages?

## Scope of the study

The main objective of this thesis was to examine the development of physiological mechanisms in the embryos of *H. edwardsii* and *H. crenulatus* related to survival of the salinity conditions on the shore, from spawning to hatching.

This objective was realized by employing the following experimental investigations, on a short term (acute) and long term basis throughout the period of development: salinity tolerance estimates, development and hatching success in low salinities, osmotic and ionic regulation at successive developmental stages, metabolic rates in

terms of oxygen consumption, mechanisms of osmoregulation, morphological and ultrastructural studies.

Chapter 2 documents the morphology and ultrastructure of the eggs[1]. Based on morphological and physiological features, developing eggs were grouped into 5 stages. This staging scheme was used in the experiments described in the following chapters in order to time physiological events and standardise treatment groups. Ultrastructural studies of these eggs provided a structural basis for the identification of potential barriers to water and solutes. The demonstration of a presumptive ion regulatory area in these eggs is reported in the same chapter. Survival rates and osmoregulatory abilities of these eggs are described in Chapter 3. It is shown that postgastrula stage eggs are more tolerant to hyposaline waters and hyperosmoregulate throughout development and in dilute seawater. Chapter 4 examines the regulation of individual ions and their contribution to osmotic regulation. Chapter 5 provides further information about the potential mechanism of osmoregulation in these eggs by analysis of $Na^+/K^+$ ATPase activity during development and when exposed to dilute seawater. Chapter 6 describes the permeability of egg membranes throughout the development of *H. crenulatus*. High turnover rates for water and sodium were observed at all developmental stages. Metabolic rates in relation to osmotic stress are documented in Chapter 7. The oxygen consumption of eggs increased during development. Metabolic rates were relatively unaffected by acute exposure to hyposaline waters. There was a metabolic cost of development in dilute seawater mainly in terms of increased development time.

[1] Some authors use the term "egg" synonymously with unfertilized ovum. In this thesis, the term refers to the extraembryonic membranes including the stalk (funiculus), the extraembryonic fluids and the zygote or developing embryo within, i.e. the whole structure attached to the female pleopods from spawning until hatching of the larva. (c.f Encyclopaedia Britannica, 1979).

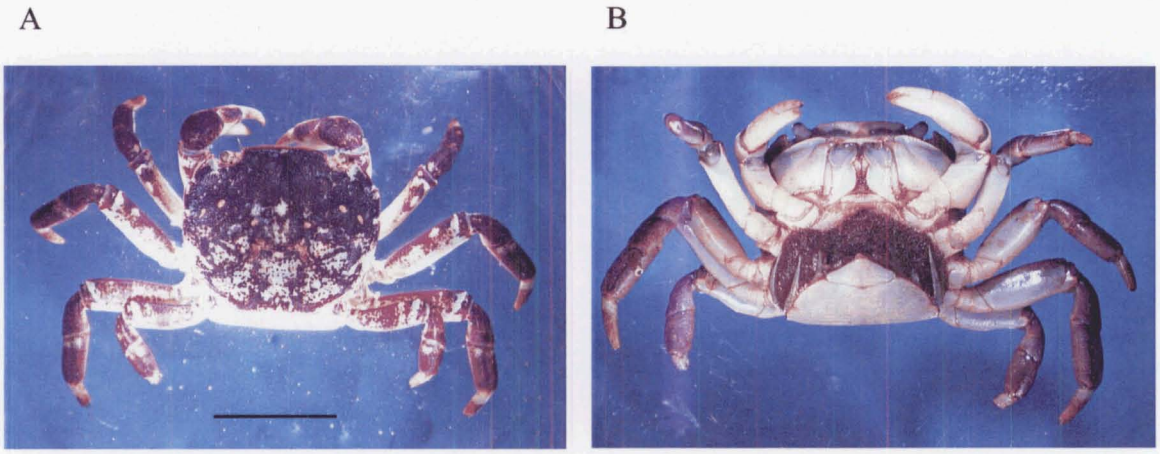



**Figure 1.1** (A) Dorsal and (B) ventral views of ovigerous female, *Hemigrapsus edwardsii*. Scale bar = 2 cm.

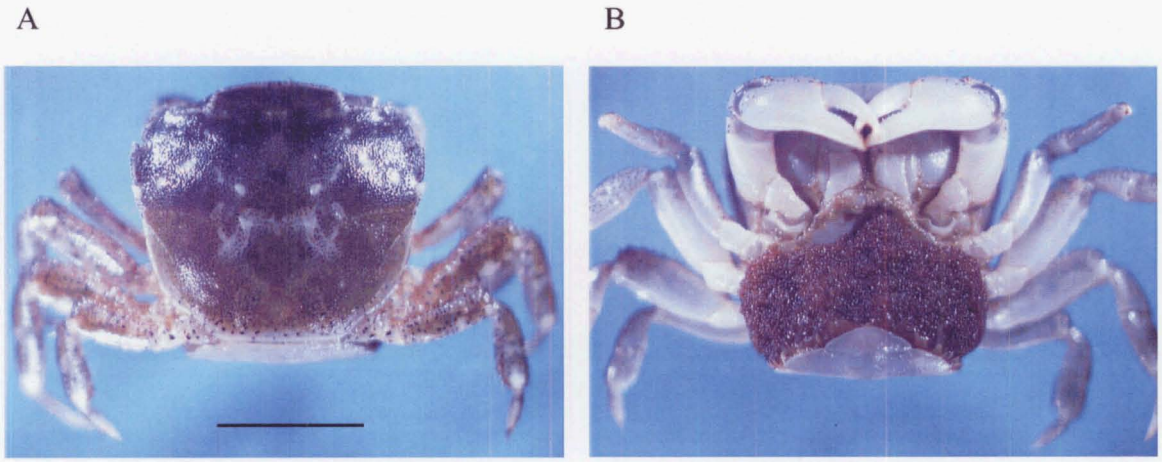



**Figure 1.2** (A) Dorsal and (B) ventral views of ovigerous female, *Hemigrapsus crenulatus*. Scale bar = 1 cm.

A

B

C

**Figure 1.3** Habitats of study animals. (A) Waipara Beach intertidal zone, the collection site for *H. edwardsii* (B) Close-up of collection site shown in (A) (within dotted area). Arrow indicates a freshwater stream which crosses the shore, ( C ) Avon-Heathcote estuary at low tide. *H. crenulatus* were trapped at high tide over the mud flats and in the channel.

# CHAPTER TWO

# MORPHOLOGY AND ULTRASTRUCTURE OF EMBRYOS


*Summary*

- At a constant temperature of 15 °C, the average incubation periods recorded for *H. edwardsii* and *H. crenulatus* were 62.0 ± 3.1 and 43.0 ± 1.8 days respectively.
- A staging scheme for embryonic development of both species was established to time physiological events. (stage 1, cleavage/blastula; stage 2, gastrula; stage 3, embryos with eye spots and chromatophores; stage 4, embryos with four lobes of yolk; stage 5, embryos with two lobes of yolk).
- Two distinct egg membranes were observed in light and electron microscopic studies in all developmental stages of both species.
- The appearance of a characteristic chloride permeable area on the surface of the eggs from gastrulation was observed by silver staining.


## INTRODUCTION

The scheme of development exhibited by crustaceans is quite different from that of most other arthropods (Anderson, 1973). Decapod crustaceans have internal fertilization and undergo direct development from yolky eggs. The basic pattern of embryonic development includes, firstly, yolk cleavages followed by invagination, gastrulation, formation of a tissue cap, and subsequent stages recognized as nauplius and metanauplius both confined to embryonic life and hatch at a zoeal stage in development.

The study of embryonic development in decapod crustaceans has frequently involved staging systems based upon particular developmental events, e.g. relative size and colour of the yolk mass, appearance of segmentation, chromatophores, heartbeat and eye development (Bas & Spivak, 2000; Boolootian *et al.*, 1959; Helluy & Beltz, 1991; Jones & Simons 1982; Lawinski & Weglarska, 1959; Leelapiyanart, 1996; Pandian, 1970; Perkins, 1972; Subramoniam, 1979; Valdes *et al.* 1991; Wear, 1974; Yamaguchi, 2001).

Steele and Steele (1975) documented that the duration of embryonic development of crustaceans increases with the increase in egg size. The size has a number of interrelated ecological implications. The size of the egg can influence the number of eggs per brood, the stage of development and the size of young when they are released to the environment (Steele & Steele, 1975). Large eggs produce large young which may improve their subsequent survival. The size of the egg is directly correlated with the amount of yolk present which in turn influences the segmentation and further development of the embryo. The rate of yolk utilization varies from species to species according to the time taken for egg development, but generally yolk is used slowly during early development and is rapidly depleted during the last few days before hatching.

The incubation period in decapod crustaceans is reported to be influenced by temperature (Wear, 1974). Wear (1974) made an extensive study of the relationship between incubation time and temperature and a reduction in the larval viability was recorded when the entire period of egg development took place in temperatures outside the normal range of their development. During embryonic development, the egg volume increases (Wear, 1974). This increase in egg volume with development has been described as osmotic uptake of water (Davies, 1981). Wear (1974) reported that the rate of egg volume increase is slower in the eggs of species with a long development period than in those which develop rapidly.

Most egg cells are surrounded by one or more membranes. The process of egg-membrane formation and the attachment of eggs to certain setae of the pleopods in decapod crustacea has been a subject of much discussion (Charmantier & Aiken. 1987; review in Cheung, 1996; Goudeau & Lachaise, 1983, Saigusa, 1992; Young, 1946). The fundamental similarities in crustacean egg membranes have been discussed in several studies (Cheung, 1966; El-sherief, 1993; Goudeau & Lachaise, 1983; Stromberg, 1972). Goudeau & Lachaise (1983) suggested that the newly laid eggs of the decapod crustaceans is enclosed in a thick capsule which is the fertilization envelope and other accessory envelopes may later develop surrounding the embryo. The presence of two distinct membranes (inner and outer) in the eggs of *Homarus americanus* toward the end of embryonic development was demonstrated by Charmantier and Aiken (1987). According to Cheung (1966), the outer

("trichromatic") membrane lacks chitin and consists of three distinct layers. The most external of these, the 'primary egg membrane" is formed in the early oocyte stage, while the other two layers are formed after extrusion and fertilization. The inner membrane consists of two layers. One is an embryonic cuticle, while the other, formed at the nauplius stage, is a cuticle resulting from an ecdysis. Yonge (1946) clearly demonstrated the presence of two non-living membranes (outer and inner) around the developing eggs of *Homarus vulgaris* and he described further that the outer membrane had restricted permeability while the chitinous inner layer was freely permeable.

During embryonic development of many crustaceans transitory organs appear, which connect the embryo with the embryonic envelopes (review Fioroni, 1980). An organ called the 'dorsal organ' which is best known in the Branchiopoda and has been variously termed a salt gland, dorsal organ, nackenorgan or nuchal organ (neck organ) is one such transitory organ that appears in developing embryos, larvae and adults (Aladin & Potts, 1995; Conte *et al.*, 1972; Ewing *et al.*, 1974; Fioroni, 1980; Hootman *et al.*, 1972; Hootman & Conte, 1975; Martin & Laverack, 1992; Meschenmoser, 1989; Morrit & Spicer, 1995). This organ is always located in the anterior region of the animal but the time of formation and eventual degeneration of this structure varies among different species. Martin and Laverack (1992) considered the embryonic dorsal organ to be a different structure from adult/larval dorsal organs. There are numerous hypotheses as to the function of this structure. According to Fioroni (1980) the function of the dorsal organ is often trophic and combined with ecdysis. Aladin and Potts (1995) described that in the malacostracans it appears to be sensory and functions suggested include chemoreception, mechanoreception and baroreception. Other studies on cladocerans, amphipods and *Artemia salina* have revealed a correlation of this structure with the process of osmoregulation and it is reported to function either as the site of salt uptake or as an ion permeable area (Conte *et al.*, 1972; Felder *et al.*, 1986; Hootman & Conte, 1975; Morrit & Spicer, 1995). The appearance of transitory osmoregulatory organs especially during embryonic development may be necessary in relation to the above mentioned functions as they have not yet developed the organs responsible for excretion and ion uptake as in adults.

The "purple rock crab", *Hemigrapsus edwardsii* is a late autumn-winter breeder with ovigerous females being recorded from March to August (Wear, 1970, Begg, 1980; Thomson, 1905). According to Begg (1980) 96% of females were ovigerous by May at Portobello, Otago. At Waipara where I collected crabs, ovigerous females of *H. edwardsii* are found from mid April to late July. As Begg reported, about 90% females were ovigerous by late May and I observed completion of egg hatching by August. The record of Wear (1970) of an ovigerous female from Otago Harbour in December 1964 therefore seems to be exceptional and may have been the result of unusual environmental conditions. Usually female ovigerous crabs were abundant in the upper intertidal zone and mainly males present towards the mid-tide area. My observations support the generally accepted view that there is a single batch of eggs each year. Bennett (1964) estimated that 26,000 eggs were carried by a female, but did not specify female size.

Compared with *H. edwardsii*, the "hairy-handed crab", *Hemigrapsus crenulatus* is a spring-summer breeder (Thomson & Anderton, 1921; Wear, 1970). Clark (1987) found that ovigerous females occurred for 8-9 months from June to January/February, with over 50% of females' ovigerous in October in the Avon-Heathcote Estuary. However, in my field study, I observed ovigerous females only from August to late January at the Avon-Heathcote Estuary and the highest percentage ovigerous was similar to that recorded by Clark (1987). Wear (1970) noted that by late November, *H. crenulatus* females at Wellington carry eggs at all stages of development. I found a similar situation in the Avon-Heathcote Estuary. Female ovigerous crabs carry eggs at different developmental stages during November to January period. Also, as noted, females lay more than one batch of eggs per season. Incubation time of about 8-12 weeks was recorded by Clark (1987). One female of 15.6 mm CW collected by Thomson and Anderton (1921) from Otago Harbour in January, carried 8,968 eggs with an average diameter of 0.26 mm.

As noted, many aspects of the ecology and reproductive biology of these two crab species, *Hemigrapsus edwardsii* and *Hemigrapsus crenulatus* have been studied (McLay, 1988). However, the physiology of developing embryos has been neglected. The overall objective of this thesis is to examine the development of physiological mechanisms associated with water and salt balance and the metabolic exchanges with

the environment. This chapter provides the structural basis for these functions and identifies potential barriers to the exchange of water and solutes. Critical events in embryonic development are documented and incorporated into a staging scheme that is used in the following chapters to time physiological events.

Localization of area/areas (embryonic tissue) possessing high ionic permeability (potential ion-transporting epithelia) in the embryo was accomplished using the modified silver nitrate staining technique described by Holliday (1990).

## MATERIALS & METHODS

### Maintenance of animals

Ovigerous crabs (female crabs carrying embryos) of *H. edwardsii* and *H. crenulatus* were collected from the intertidal zones at Waipara (Figure 1.3) and at the Avon-Heathcote Estuary (Figure 1.3) respectively during the period of spawning. Preliminary microscopical studies were carried out to identify embryos at different stages of development.

For studies of the timing of development, 10 crabs of each species carrying just spawned embryos were labelled by gluing (cyanoacrylate) small numbered plastic tags to the carapace and reared in a recirculating seawater system in controlled temperature (15 $^{o}$C) under a 12h light and 12h dark cycles and simulated tidal emersion (Appendix A). The salinity of seawater was maintained at 35‰ (1000 ± 5 mOsmol.kg$^{-1}$) throughout the study period. The crabs were fed mussel and the seawater was changed completely every fortnight.

Other morphological studies were carried out on embryos removed from ovigerous crabs collected with embryos at a range of developmental stages and maintained in a recirculated seawater aquarium of the Department of Zoology (15 $^{o}$C, 35‰).

### Embryonic development

Based on morphological and physiological features, the timing of development of embryos was determined.

To examine the embryonic development and incubation time, a sample of 5 embryos from each of the 10 ovigerous crabs reared in the tidal system tank was picked off with a pair of fine forceps at the start of the experiment, after 0.5 days, 1 day, 3 days and thereafter at 3 day intervals until the embryos hatched. The morphological features noted were: changes in relative size of the embryo, yolk content, embryonic

pigmentation, eye development and heart beat. Photographs of embryos at different stages of development were taken using a camera mounted on a stereo-microscope to categorise different stages.

To determine egg volume, 25 embryos from the 10 crabs were detached at each sampling time as above. The largest (D) and the smallest (d) diameters of the sampled embryos were measured with a stereomicroscope equipped with a micrometric eye-piece, and egg volume (V) was calculated assuming an ellipsoidal formula ($V = d^2 \times D \times \pi/6$). Percentage yolk remaining in different developmental stages was estimated approximately in relation to the total egg volume.

**$AgNO_3$ staining of embryos**

Embryos were stained with $AgNO_3$ using a method based on that of Holliday (1990). Eggs containing embryos at different developmental stages were detached from the ovigerous crabs maintained in the aquarium room and first rinsed for 30 s in each of three changes of distilled water to remove adherent chloride ions. The specimens were then transferred to 0.5% $AgNO_3$ for 5 min and rinsed in distilled water three times as before. Some were left in distilled water and others returned to seawater (in order to reduce cellular degeneration) and exposed to sunlight for 10-15 min until the appearance of a black-brown AgCl precipitate. In some cases, the stained areas were intensified by immersion in Kodak D-19 developer, an alternative procedure used by some workers, (Lindhjem *et al.*, 2000; Dickson *et al.*, 1991). $AgNO_3$ staining was also performed on hatching larvae.

Light microscope (Zeiss Stemi 2000-c) observations were made without delay on these stained, unfixed embryos and photographs were taken using a Digital Still Camera (Sony DSC-S75) mounted on the microscope.

**Scanning Electron Microscopy**

$AgNO_3$ stained embryos and unstained control embryos were viewed using the scanning electron microscope (SEM). All developmental stages of both *H. edwardsii* and *H. crenulatus* were examined.

Embryos were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 0.3 M sucrose (pH 7.3) for at least 48 h at 4 °C. Following rinsing in buffer overnight, the samples were dehydrated in a graded alcohol series (30%, 50%, 70%, 80%, 90%, 95% and 100% ethanol, about 2 h in each solution, with a final period of 18 h in 100% ethanol). Samples were then transferred to amyl acetate after passing them through a 4-step absolute ethanol/amyl acetate series (25%, 50%, 75% and 100% amyl acetate, 2 hours in each solution and overnight in a fresh solution of 100% amyl acetate). After drying in a liquid $CO_2$ critical-point drier, the specimens were mounted on 1.0 cm diameter aluminium stubs using carbon impregnated double-sided Sellotape tabs. In order to view the $AgNO_3$ deposit, the outer egg membrane was then carefully ruptured with the tip of a fine scalpel blade and the egg manoeuvred with forceps to a new area of the Sellotape for secure attachment. Where possible, the stained patch was positioned uppermost for best observation.

Finally, the samples were sputter coated with 60 nm of gold/palladium and observed with a Leica S440 SEM at an accelerating voltage of 15 kV. The images were collected digitally and processed with Adobe Photoshop 5.0.

**X-ray Microanalysis of the $AgNO_3$ stained area**

The nature of the reaction between $AgNO_3$ and the embryos was further examined by performing energy dispersive x-ray microanalysis on the stained patch area. The x-rays were collected with a Link Pentafect detector fitted to the same SEM as above and using Link ISIS Software 3.34. An accelerating voltage of 20kV was used together with a probe current of 600 pA. The specimens were analysed at 1000x using the spot analysis mode. Spectra were generated (2 min live time) and peaks

representing various elements were identified. X-ray dot mapping of embryos was conducted to show the distribution of silver within the patch area.

**Transmission Electron Microscopy**

Many attempts were made to develop a successful protocol for the transmission electron microscopy of embryos of *H. edwardsii* and *H. crenulatus*. Due to the high yolk content of these embryos and the presence of membrane layers, that appeared to be relatively impermeable to fixation chemicals, fixation and embedding proved to be extremely difficult. After exhaustive trial and error, the following version of the schedule gave best results for these embryos.

Embryos at each of the 5 developmental stages were placed in the primary fixative (3% glutaraldehyde, 1.2% paraformaldehyde, 0.05% tannic acid, 0.05% saponin, 0.1 M sodium cacodylate buffer, made up using 50% sea water, pH adjusted to 7.3). The osmolality of the fixative was determined using a vapour pressure osmometer (Wescor 5500) to be ca. 1300 mOsm/kg, slightly above the egg osmolality. Initial fixation was for 1 h at room temperature. Then the samples were placed in a vacuum desiccator for a further 1.5 h at room temperature. The fixative was changed with fresh solution and fixation continued overnight at 4 °C. The samples were washed with two 10 min rinses in 0.1 M sodium cacodylate (made up in 70% seawater), followed by the same buffer made up in 50% seawater and finally in 0.2 M sodium cacodylate buffer in distilled water (three 10 min washes).

Post-fixation with 1% osmium tetroxide ($OsO_4$) in 0.2 M sodium cacodylate in distilled water was carried out for 4 h at 4 °C, followed by three 10 min buffer washes (0.2 M sodium cacodylate in distilled water). Then the embryos were dehydrated in a graded ethanol series: 30%, 50%, 70% (3 changes) 80%, 90%, 95% and 100% (3 changes), for 20 min in each solution.

Infiltration of resin (Spurr, 1969) was achieved using a graded series of ethanol/propylene oxide (3:1,1:1,1:3, pure – 20 min in each with 3 exchanges in the pure chemical) and then a propylene oxide/resin series. Problems associated with the

floating of embryos in propylene oxide/resin solutions resulted in a long, slow procedure. Resin was added drop by drop and halted when embryos did not remain submerged. The sample was then put on a rotator at 16 rpm for 20 min. Further resin was added until the ratios 3:1, 1:1, 1:3, 1:8, pure resin was achieved. At each concentration, the embryos were rotated for 12 h. Embryos that did not remain submerged in the solutions were discarded at the next solution change. Some specimens were placed in a vacuum desiccator for 1.5 h at room temperature once they were in pure resin.

All embryos were then polymerised in fresh resin in plastic moulds at 65 °C for 18 h. Following polymerisation, semithin (1-2 μm) and ultra-thin sectioning (80-90 nm) was accomplished with a Leica UCT ultramicrotome, using both glass and diamond knives. Ultra-thin sections were mounted on 200 mesh copper grids and stained with 5% uranyl acetate (in distilled $H_2O$) for 30 min followed by Sato's triple lead stain for 20 min. The sections were examined with a JEOL JEM1200-EX transmission electron microscope at an accelerating voltage of 80kV. Images were photographed using Kodak large format plate negatives (# 4489).

**Light microscopy with semithin sections**

Semithin (1 or 2 μm) sections from the resin-embedded specimens were mounted on glass slides and stained with 1% toluidine blue in a 1% borax solution. Observations were made using a Zeiss Axioskop 2 Mot microscope and images were captured with the Zeiss software Axiovison 3.1.

# RESULTS

## General morphology of embryos

*Hemigrapsus edwardsii*

Newly laid embryos of *H. edwardsii* were easily deformable and ellipsoidal in shape (Figure 2.1). During development they became more spherical. Mean diameters of newly laid embryos were 0.34 x 0.33 mm and reached 0.41 x 0.40 mm just before hatching (Table 2.1). The volume of single egg increased during development. The mean incubation time from spawning to hatching was 62.0 ± 3.1 days at 15 °C (n = 10 crabs). The colour of the embryo changed from orange-red at the beginning to colourless with the depletion of yolk (Figure 2.1).

*Hemigrapsus crenulatus*

At 15 °C, the embryos were incubated for a period of 43.0 ± 1.8 days (n = 10 crabs). As for *H. edwardsii*, newly laid embryos were deformable and ellipsoidal and attained more spherical shape with development (Figure 2.2). The embryos of *H. crenulatus* were much smaller with those of *H. edwardsii* (0.248 x 0.234 mm for newly laid embryos; 0.330 x 0.324 mm for embryos close to hatching, Table 2.2). As for *H. edwardsii*, the colour of the embryo changed from orange to colourless with development as a result of the yolk reduction (Figure 2.2).

## Developmental stages of embryos of *Hemigrapsus edwardsii*

Based on different morphological and physiological characters, I defined five different developmental stages for the embryos of *H. edwardsii*. The sequence of events observed along the embryonic development of these embryos was cleaving of yolk after a few hours or days, followed by invagination, gastrulation, tissue/organ formation and heart beat (Figure 2.1).

**Table 2.1** Mean (± SEM) egg diameters, egg volumes and development times at different stages of development of embryos of *H. edwardsii*, at 15 °C.

| Stage | Egg diameters (mm) greatest | smallest | Egg volume (nL) | Duration of stage (d) | Incubation time to end of stage (d) |
|---|---|---|---|---|---|
| 1. Cleavage to blastula | 0.345 ± 0.002 | 0.329 ± 0.002 | 19.7 ± 0.36 | 4.7 ± 0.35 | 5 |
| 2. Gastrula | 0.369 ± 0.002 | 0.356 ± 0.003 | 24.7 ± 0.59 | 23.3 ± 1.04 | 28 |
| 3. Eyespot & chromatophores | 0.392 ± 0.001 | 0.379 ± 0.001 | 29.6 ± 0.26 | 9.1 ± 0.52 | 37 |
| 4. Yolk in 4 lobes | 0.403 ± 0.001 | 0.388 ± 0.002 | 31.9 ± 0.29 | 13.1 ± 0.55 | 50 |
| 5. Yolk in 2 lobes | 0.414 ± 0.001 | 0.402 ± 0.002 | 35.1 ± 0.35 | 11.8 ± 0.56 | 62 |

**Table 2.2** Mean (± SEM) egg diameters, egg volumes and development times at different stages of development of embryos of *H. crenulatus*, at 15 °C.

| Stage | Egg diameters (mm) greatest | smallest | Egg volume (nL) | Duration of stage (d) | Incubation time to end of stage (d) |
|---|---|---|---|---|---|
| 1. Cleavage to blastula | 0.248 ± 0.003 | 0.234 ± 0.003 | 7.1 ± 0.23 | 2.2 ± 0.24 | 2 |
| 2. Gastrula | 0.279 ± 0.007 | 0.268 ± 0.006 | 10.6 ± 0.06 | 15.8 ± 0.5 | 18 |
| 3. Eyespot & chromatophores | 0.309 ± 0.009 | 0.301 ± 0.001 | 14.8 ± 0.12 | 8.8 ± 0.34 | 27 |
| 4. Yolk in 4 lobes | 0.322 ± 0.006 | 0.318 ± 0.001 | 17.2 ± 0.09 | 8.5 ± 0.4 | 36 |
| 5. Yolk in 2 lobes | 0.330 ± 0.001 | 0.324 ± 0.001 | 18.3 ± 0.12 | 7.7 ± 0.44 | 43 |

Stage 1 - **Cleavage to blastula stages** (1-5 days)

Newly-deposited embryos just after spawning were soft and easily deformed. The zygote was completely filled with yolk occupying 100% of its volume. The yolk appeared as a mass of undifferentiated material and structures were not discernible. The zygote started cleaving into cells in less than 2 days distributing yolk evenly among the blastomeres, finally reaching the blastula stage after 5 days. The average greatest and smallest diameters of the embryos of this stage were 0.345 and 0.329 mm respectively. The total egg volume was calculated as 19.7 nL for this stage.

Stage 2 - **Gastrula** ( 6-28 days)

The beginning of this stage was marked by the appearance of a small yolk-free portion (a germinal disc), corresponding to the first evidence of the embryo. Gastrulation continues further increasing the yolk-free portion and by the end of this stage a clear tissue portion was present. This stage corresponds to a rather long period of development (23 days) during which relatively minor changes in the appearance of the embryo were visible at low magnification. The egg volume was 24.7 nL for this stage (diameters of egg were 0.369 and 0.356 mm).

Stage 3 - **Eyespot & chromatophores** ( 29-37 days)

Following gastrulation, the start of this stage was defined as the appearance of two thin crescent shaped red eyespots and was characterised by readily observable organogenesis. Chromatophores started to appear in the abdominal area during this period and appendage buds were recognizable. The heart beat commenced towards the end of this stage, but was not rhythmic. The yolk was reduced but was not divided into lobes. Total egg volume increased (mean volume was 29.6 nL). This stage was completed in about 37 days.

Stage 4 - **Yolk in 4 lobes** ( 38-50 days)

The beginning of this stage was marked by the separation of the yolk into 4 large lobes. The heart beat was clear and rhythmic and movement of appendages was visible. At the end of this stage, yolk was seen as 4 more distinct lobes, 2 large and 2 small (butterfly shaped). The eyes were more developed and rounded. This stage was completed after about 50 days. Egg diameters were 0.403 x 0.388 mm and the average volume of the embryos for this stage was 31.9 nL.

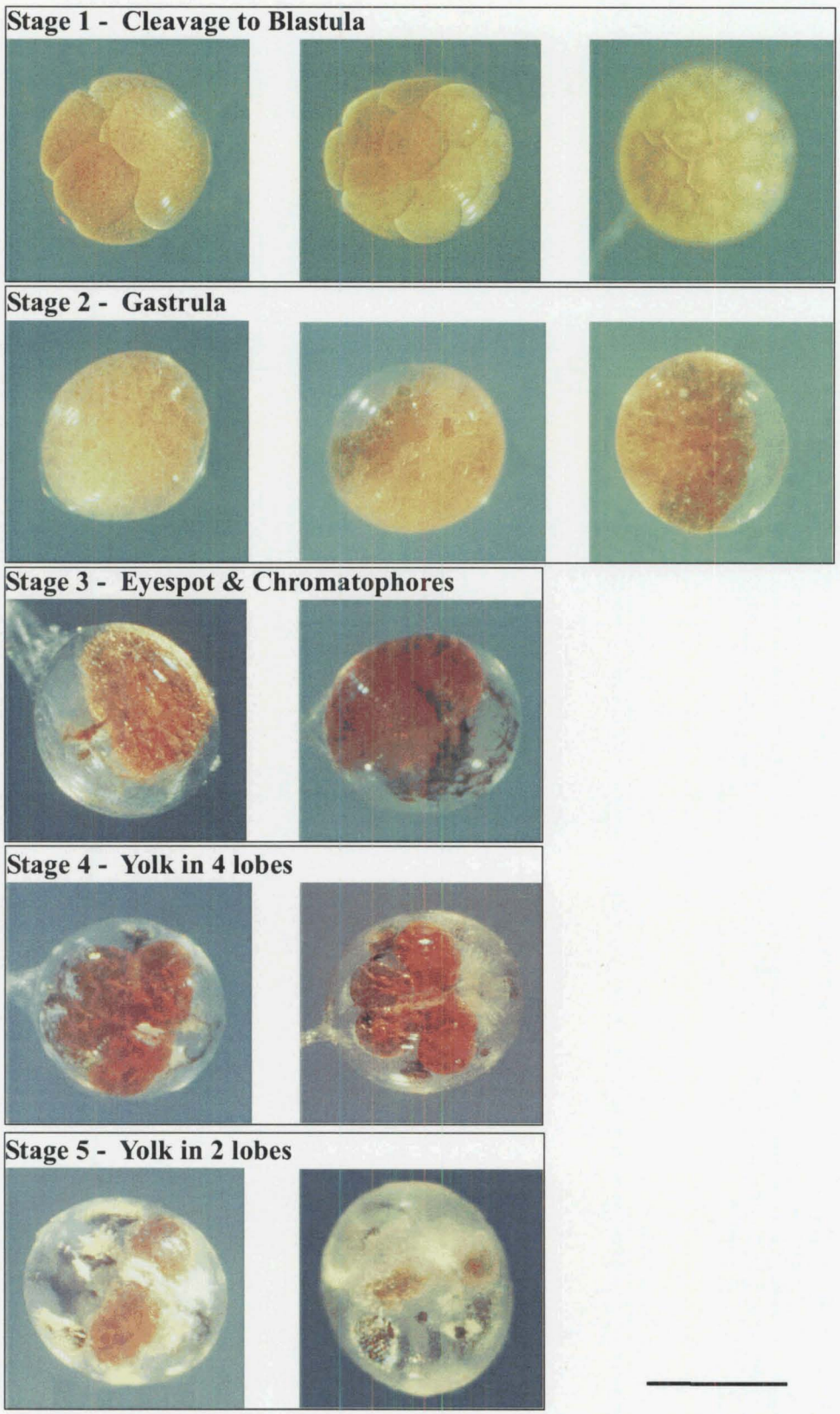



**Figure 2.1** Representative micrographs of the embryonic development of eggs of *Hemigrapsus edwardsii*, taken near the beginning and end of each stage at 15 °C. Scale bar = 300 μm.

Stage 5 - **Yolk in 2 lobes** ( 51- 62 days)

This stage commenced with the appearance of yolk as two small separate masses. The heart beat became faster and embryonic larval movement was visible. Black eyes and chromatophores were entirely formed. The yolk was reduced to two very small lobes in the colourless pre-hatching embryo. At the end of this stage, the embryo occupied almost the entire volume of the egg. During this period, total egg volume increased to 35.1 nL (diameters 0.414 X 0.402 mm). This stage ends at hatching of the embryo as a zoea.

**Developmental stages of embryos of *Hemigrapsus crenulatus***

As for *H. edwardsii*, the embryonic developmental stages of *H. crenulatus* were defined into 5 stages using the same criteria as above (Figure 2.2). The pattern of embryonic development, and the morphological and physiological observations were similar.

Stage 1 - **Cleavage to blastula** ( 0-2 days)

This stage was similar to that found in *H. edwardsii*, but it took only 2 days to complete the stage. As for *H. edwardsii*, the embryos at this stage were completely filled with yolk and the yolk cleaved into cells within a short period of time (< 0.5 days) reaching blastula in 2 days. These embryos were more soft and delicate compared to *H. edwardsii*.

Stage 2 - **Gastrula** ( 3-18 days)

As in *H. edwardsii*, this was the longest stage (16 d) and commenced with the process of invagination. The mean egg volume for this stage was 10.6 nL.

Stage 3 - **Eyespot & chromatophores** ( 19-27days)

The total duration time of this stage was about 9 days. As for *H. edwardsii*, organogenesis appeared to start in this stage. There was a rapid increase in the total egg volume during this period (14.8 nL).

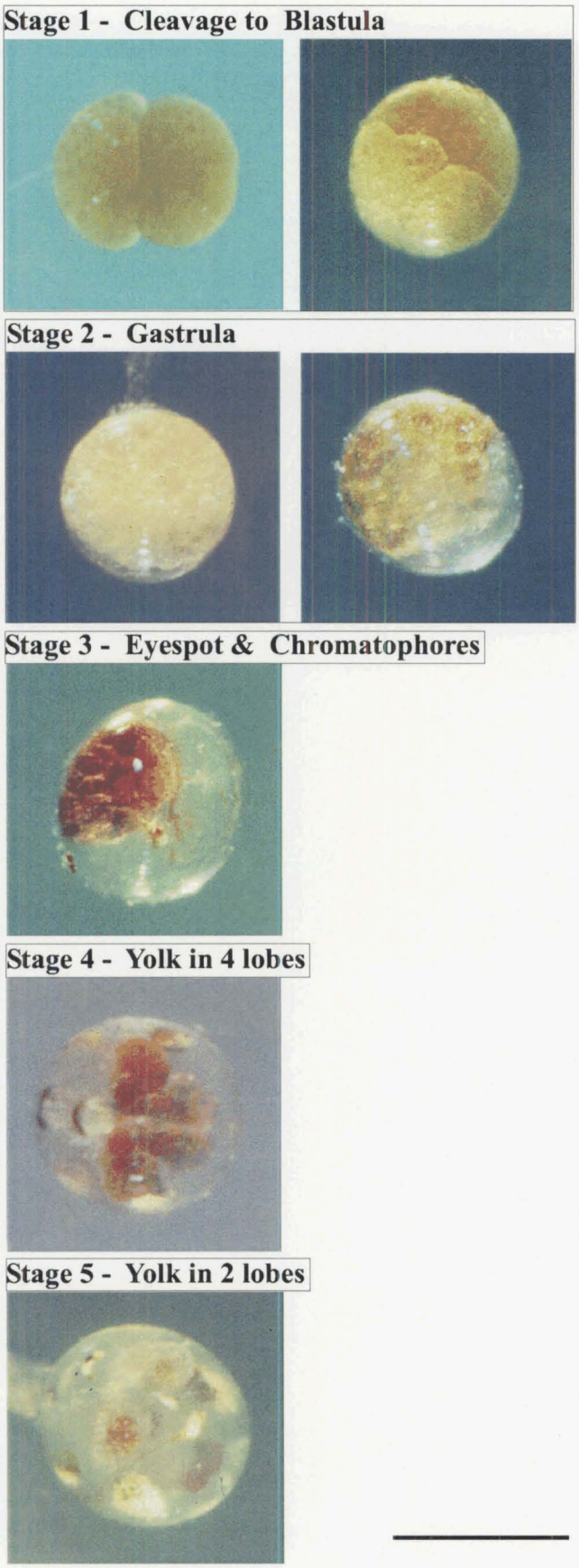



**Figure 2.2** Representative micrographs of the embryonic development of eggs of *Hemigrapsus crenulatus* at each stage at 15 °C. Scale bar = 300 µm.

Stage 4 - **Yolk in 4 lobes** ( 28-36 days)

This stage commenced with the appearance of the yolk as four lobes and the butterfly shape of the yolk was clear at the end of this stage as for *H. edwardsii*. Except for the duration of this stage, which was about 8 days, other features were the same as *H. edwardsii*.

Stage 5 - **Yolk in 2 lobes** ( 37-43 days)

As for *H. edwardsii*, this is the last stage of development and it took about 7 days to complete. The pre-hatching embryo occupied the total volume of the egg (18.3 nL) as for *H. edwardsii*.

**Silver staining of embryos *H. edwardsii* and *H. crenulatus***

The deposition of silver chloride as a characteristic surface patch was seen in light and scanning electron microscopic observation of all developmental stages of both *H. edwardsii* and *H. crenulatus*, except stage 1. This patch was always positioned on the yolk side of the egg at the opposite pole from the germinal disc or embryo (Figures 2.3 & 2.4). It was generally an oval shape and often had a darker rim. There was no discrete area of staining in the cleavage/blastula stage but smaller spots of variable size and intensity were visible over the whole surface of the egg (Figures 2.3 and 2.4).

When embryos close to hatching were stained, some embryos hatched during processing. In these cases, the stained patch was removed with the egg membranes and no sign of the deposition was seen on the hatched larvae (Figure 2.5). No such special tissue or structure was seen in the hatched larvae stained with silver nitrate observed either by light or scanning electron microscopy (Figure 2.5).

Semithin sections taken from the silver nitrate stained embryos prepared for transmission electron microscopy showed that this deposition was interposed between the outer and inner membranes of the egg (Figure 2.6). This was confirmed by the scanning electron microscopic studies of these stained embryos. In order to view the patch, it was necessary to remove the outer membrane as explained in the Materials

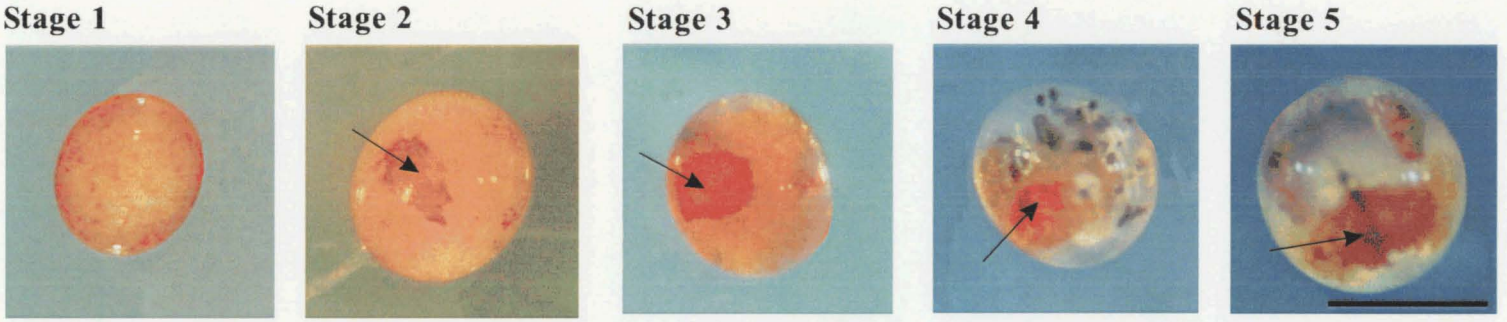



**Figure 2.3** Light microscope micrographs of silver nitrate stained embryos of *H. edwardsii* at different developmental stages. Arrow indicates the dark staining (patch) of silver deposition. Scale bar = 300 µm.

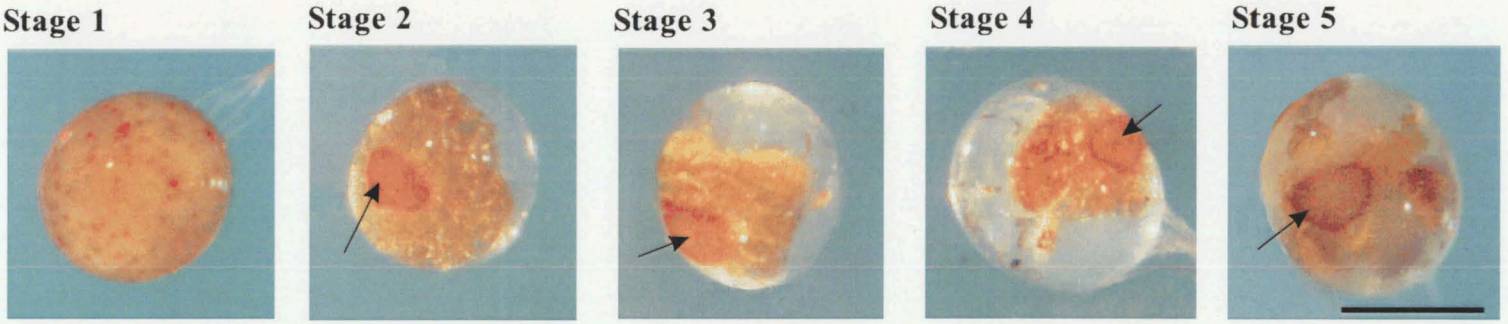



**Figure 2.4** Light microscope micrographs of silver nitrate stained embryos of *H. crenulatus* at different developmental stages. Arrow indicates the dark staining (patch) of silver deposition. Scale bar = 300 µm.

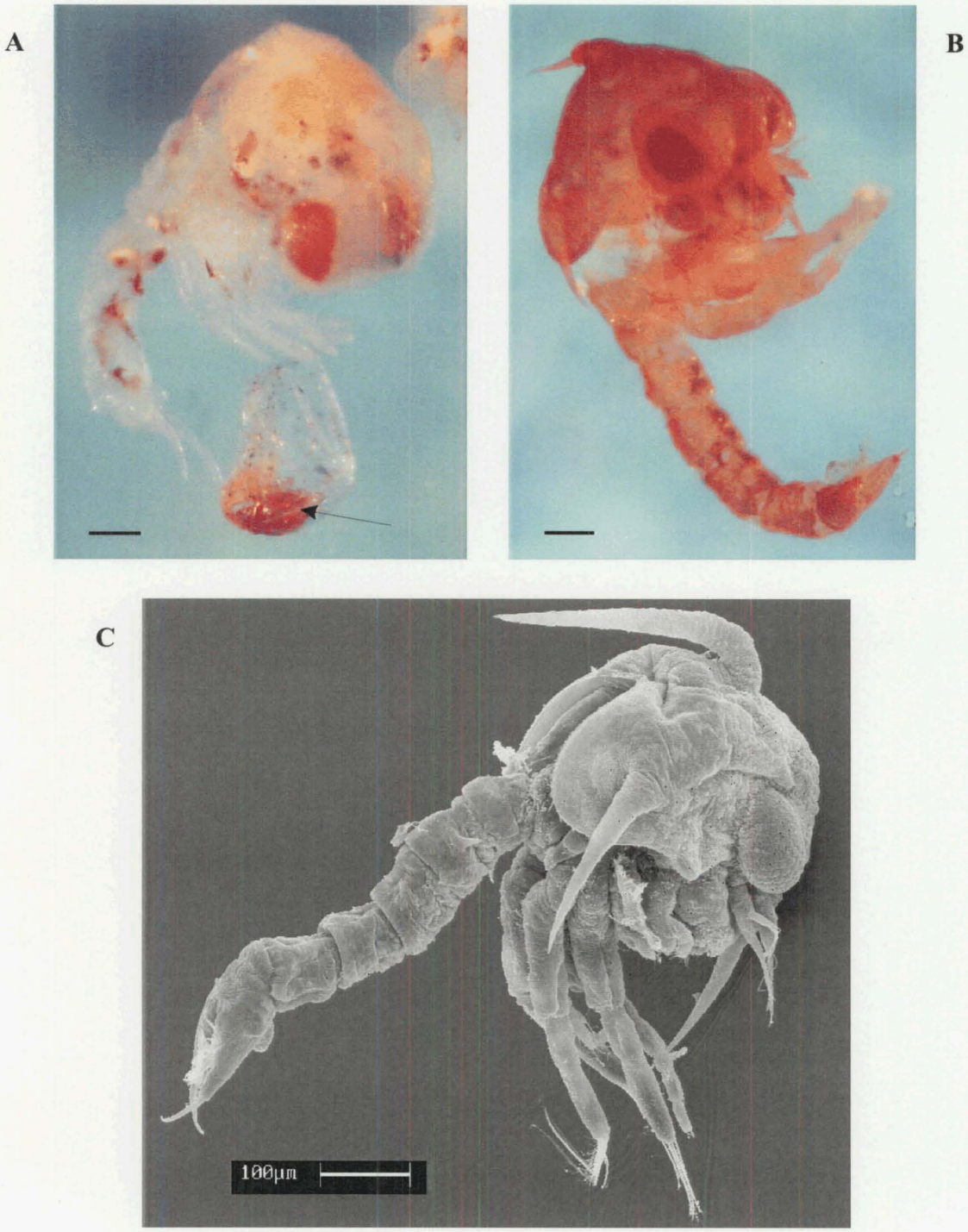



**Figure 2.5** Light microscope and scanning electron micrographs of the larvae of *Hemigrapsus edwardsii*. (A) Arrow indicates the stained patch which was removed with the egg membranes in the hatching larva (when stage 5 embryos, close to hatch, were stained with $AgNO_3$, spontaneous hatching of larvae in some embryos was observed) (B) Hatched larva stained, no obvious patch seen, & (C ) SEM micrograph of unstained hatched larva. Scale bars = 100 µm.

and Methods section (Figure 2.7). No trace of the silver deposit was present on the external surface of the egg.

Scanning electron microscope micrographs of the silver nitrate stained embryos of both *H. edwardsii* and *H. crenulatus* are illustrated in Figures 2.8 and 2.9. A distinct patch of silver chloride was deposited on the inner membrane of embryos at the developmental stages 2, 3, 4 and 5. Many small patches over the entire egg were observed in stage 1 embryos of both species, resulting in a "spotty" nature of the Ag deposits (Figures 2.8 A & 2.9 A). A transitional appearance, showing both a dark staining area and distributed spots of staining, was observed in early gastrulae (Figure 2.10).

Energy dispersive x-ray spectra of this stained patch are represented in Figure 2.11 and confirm this precipitate as silver chloride. The elemental mapping recognized silver and chloride in the stained patch and the spectrogram showed prominent peaks of silver and chloride in the energy range 2-4 keV, which corresponds to the characteristic X-rays of silver and chloride.

Figure 2.12 shows semithin sections taken from the embryos of *H. edwardsii* at stage 4 (yolk in 4 lobes) of development. These sections show the two egg membranes (inner and outer), funiculus, perivitelline space, yolk mass and the developing embryo. Also, these sections indicate the presence of some cellular material associated with the inner membrane/embryonic tissue on the yolk side of the egg in the region where silver is deposited. The actual relationship of the membranes and embryonic tissue was difficult to determine at times due to possible artefacts associated with swelling and shrinkage of the egg during the microscopy processing.

**Transmission Electron Microscope micrographs - egg membranes**

The presence of two distinct membranes (outer & inner) in the embryos of *H. edwardsii* at the stage 4 of development is demonstrated in Figure 2.13. This observation was consistent for all postgastrula stages of both species (Stage 1 embryos were not viewed). The outer egg membrane consisted of 3 layers and the

thickness of these layers varied in different embryos and positions around the egg. The third layer of the outer membrane, close to the inner membrane, appeared to be cellular (or composed of cell debris) and seemed to be the most variable in thickness. The inner membrane was a singular layer and was less electron-dense than the outer membrane. It closely adhered to the outer membrane in some places (Figure 2.12). The funiculus (or stalk) for attachment of embryos to the female pleopods is formed of the outermost layer of the outer membrane (1L).

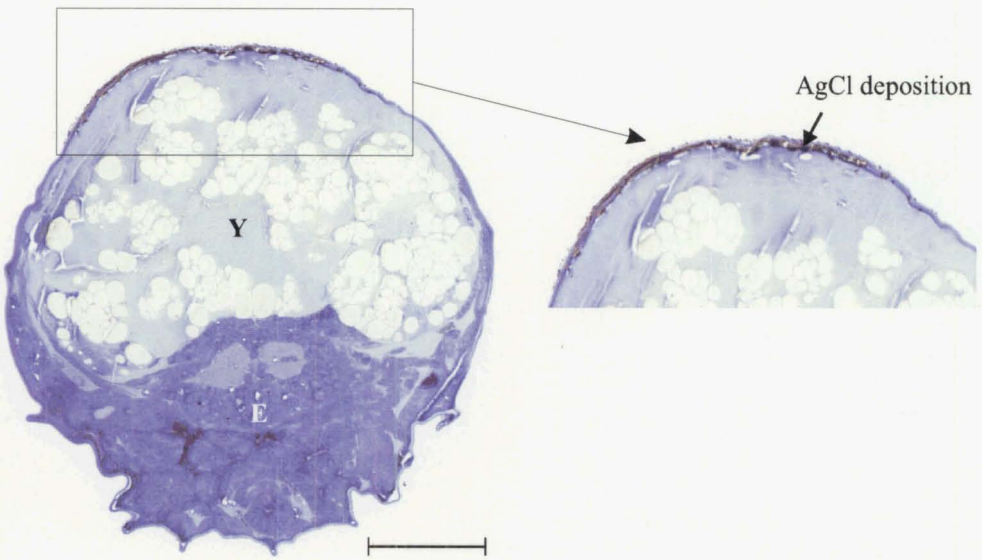



**Figure 2.6** Semithin section of the $AgNO_3$ stained embryo of *H. edwardsii* at stage 4 of development (Yolk in 4 lobes), showing that the AgCl deposition is in between inner and outer membranes. Y = yolk, E = embryo. Scale bar = 50 μm.

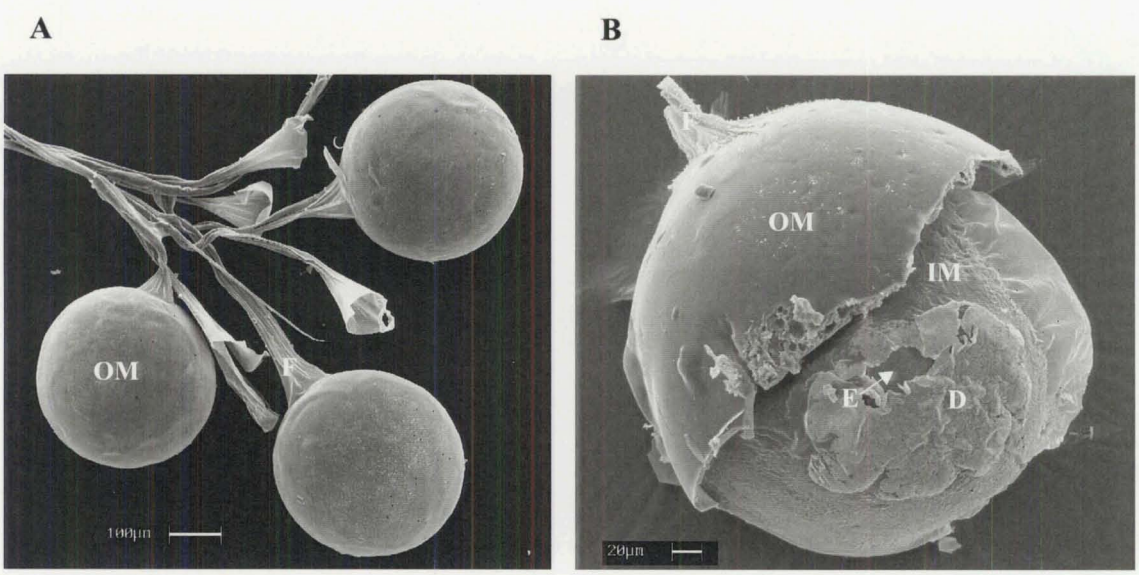



**Figure 2.7** Scanning electron microscope micrographs of the $AgNO_3$ stained embryos of *H. edwardsii* at stage 4 of development (Yolk in 4 lobes). (A) With outer membrane intact, (B) Outer membrane half removed to show the patch is on the inner membrane. D = silver chloride deposition, E = embryo, F = funiculus (stalk), IM = inner membrane & OM = outer membrane.

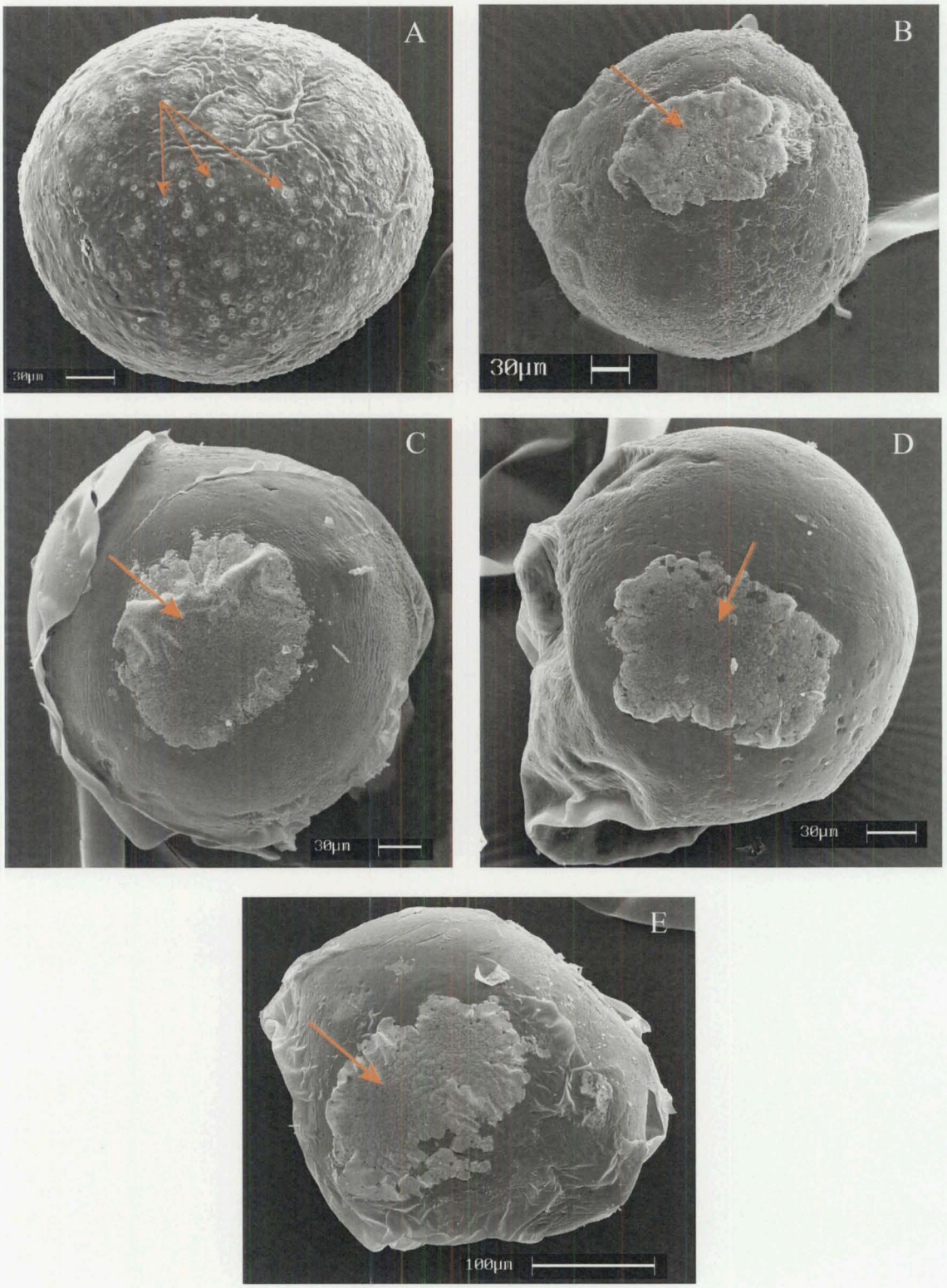



**Figure 2.8** Scanning electron microscope micrographs of $AgNO_3$ stained embryos of *H. edwardsii* at different developmental stages. The outer membrane has been completely or partially removed. Arrows indicate the deposition of AgCl on the inner membrane. (A) Stage 1, (B) Stage 2, (C) Stage 3, (D) Stage 4 & (E) Stage 5.

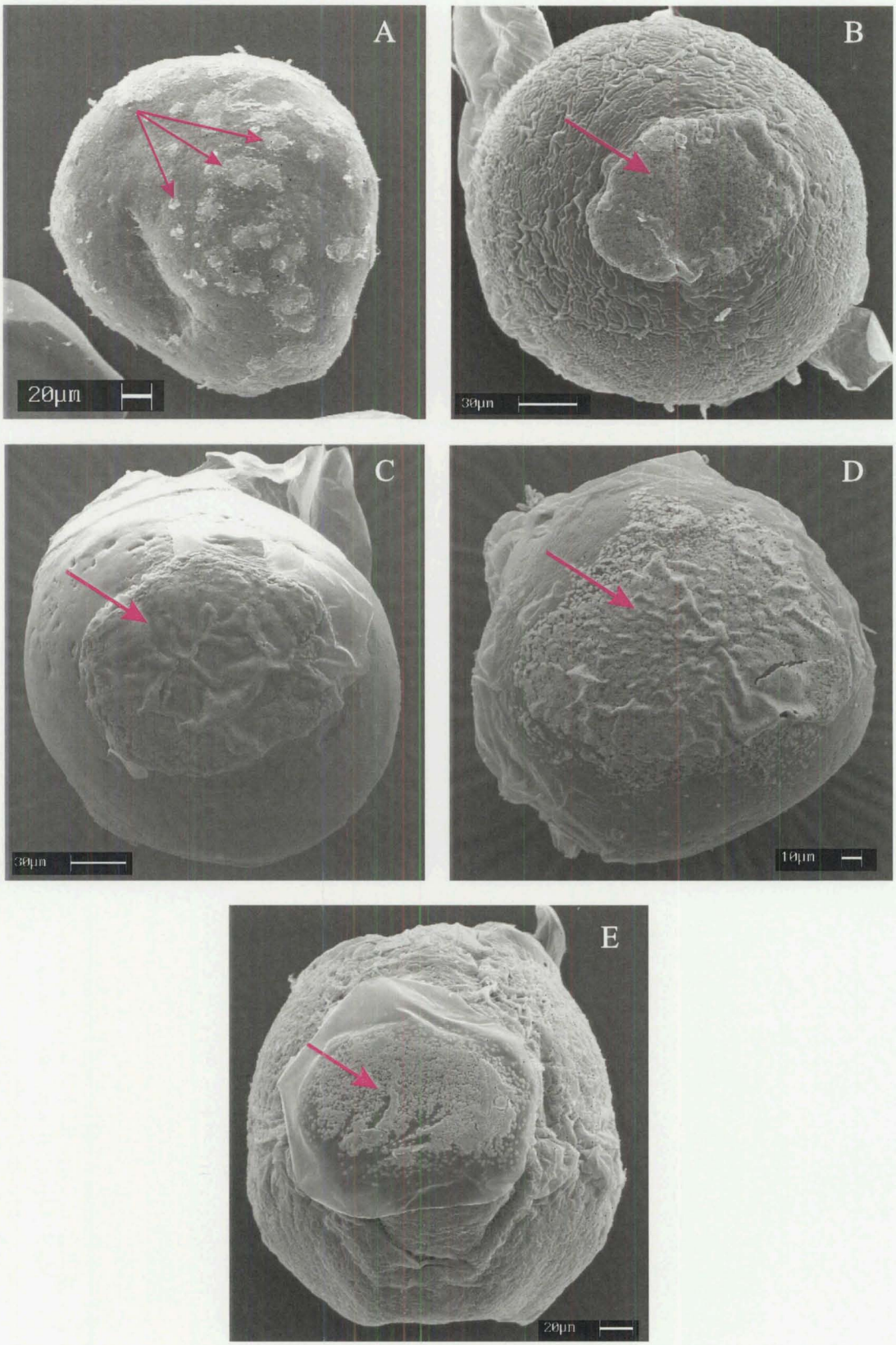



**Figure 2.9** Scanning electron microscope micrographs of $AgNO_3$ stained embryos of *H. crenulatus* at different developmental stages. The outer membrane has been completely or partially removed. Arrows indicate the deposition of AgCl on the inner membrane. (A) Stage 1, (B) Stage 2, (C) Stage 3, (D) Stage 4 & (E) Stage 5.

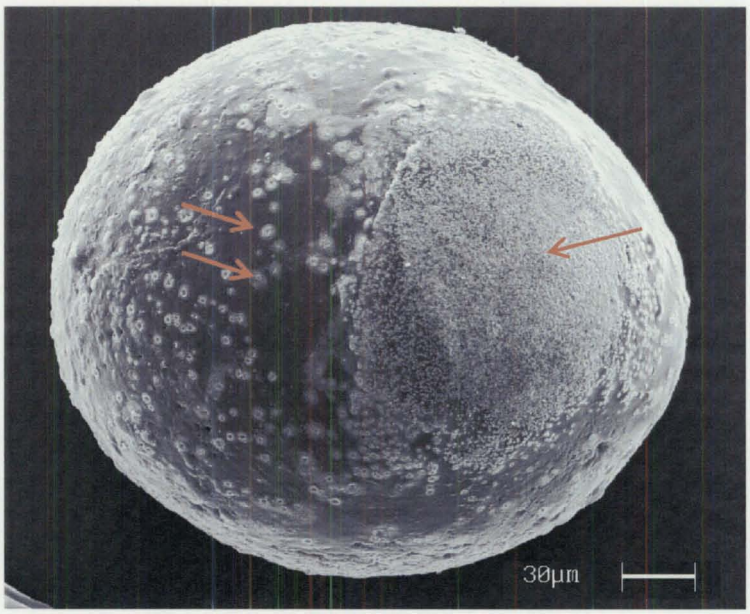
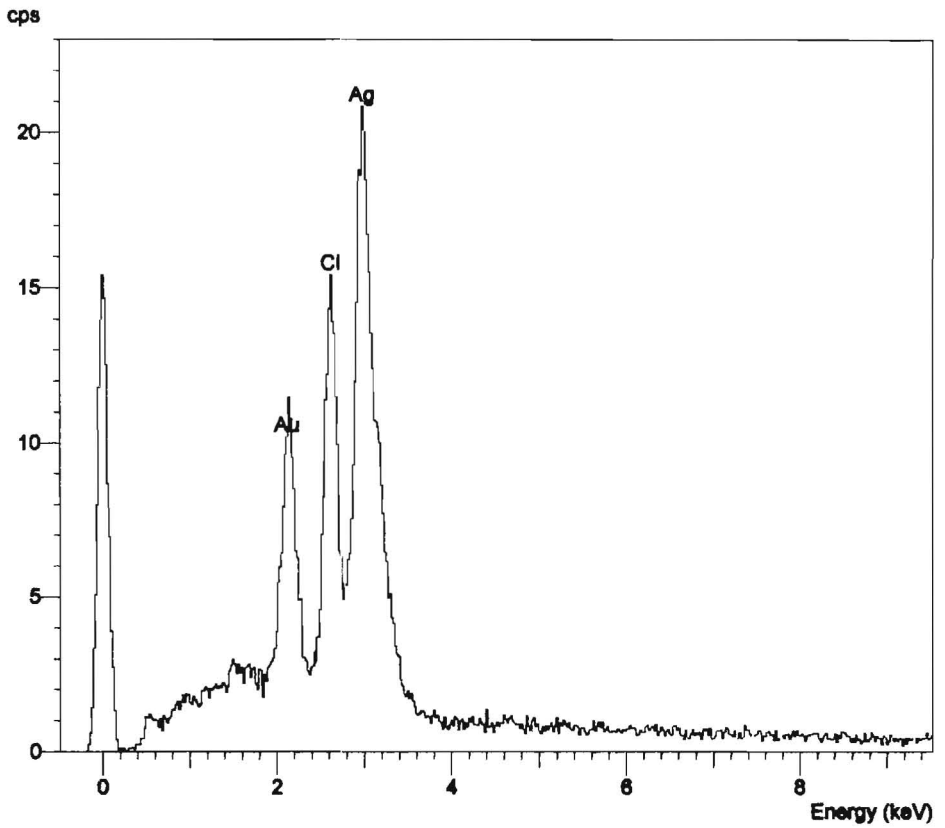



**Figure 2.10** SEM micrograph showing the silver chloride deposition as a characteristic patch and as several small "spots" on the inner membrane of early gastrula stage embryo of *H. edwardsii.*

(A)




(B)

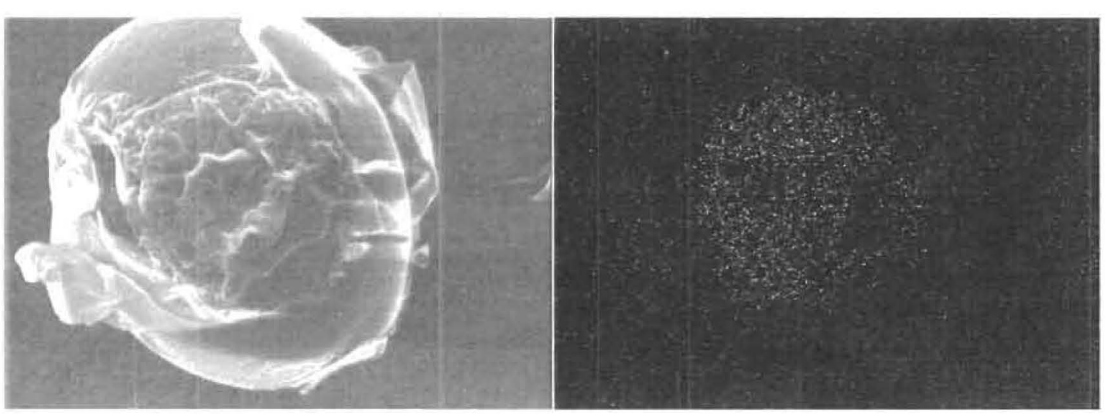


**Figure 2.11** X-ray microanalysis of the $AgNO_3$ stained area on the embryo of *H.edwardsii* at stage 4 of development. (A) Representative spectrogram of the stained area showing Ag and Cl as major constituents in the stained patch (Au peak is due to sputter coating of gold onto the sample to increase conductivity), (B) Elemental dot mapping to show the localization of silver on the stained area.

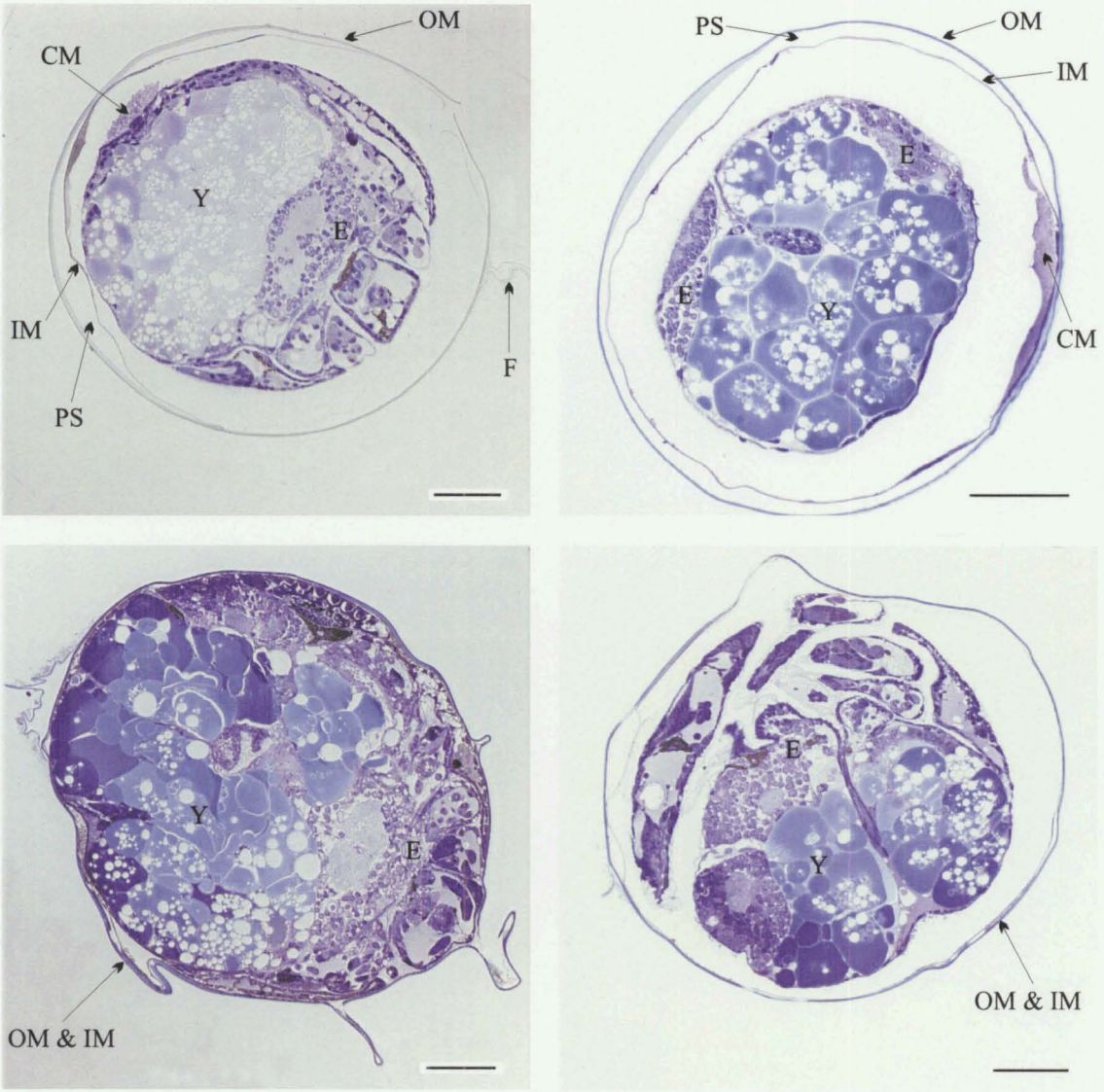



**Figure 2.12** Semithin sections of the embryos of *H. edwardsii* at stage 4 of development (yolk in 4 lobes) showing egg membranes, cellular layer attached to membranes, developing embryo & the yolk. CM = cellular material, E = embryo, F = funiculus, IM = inner membrane, OM = outer membrane, PS = perivitelline space, Y = yolk. Scale bars = 50 μm.

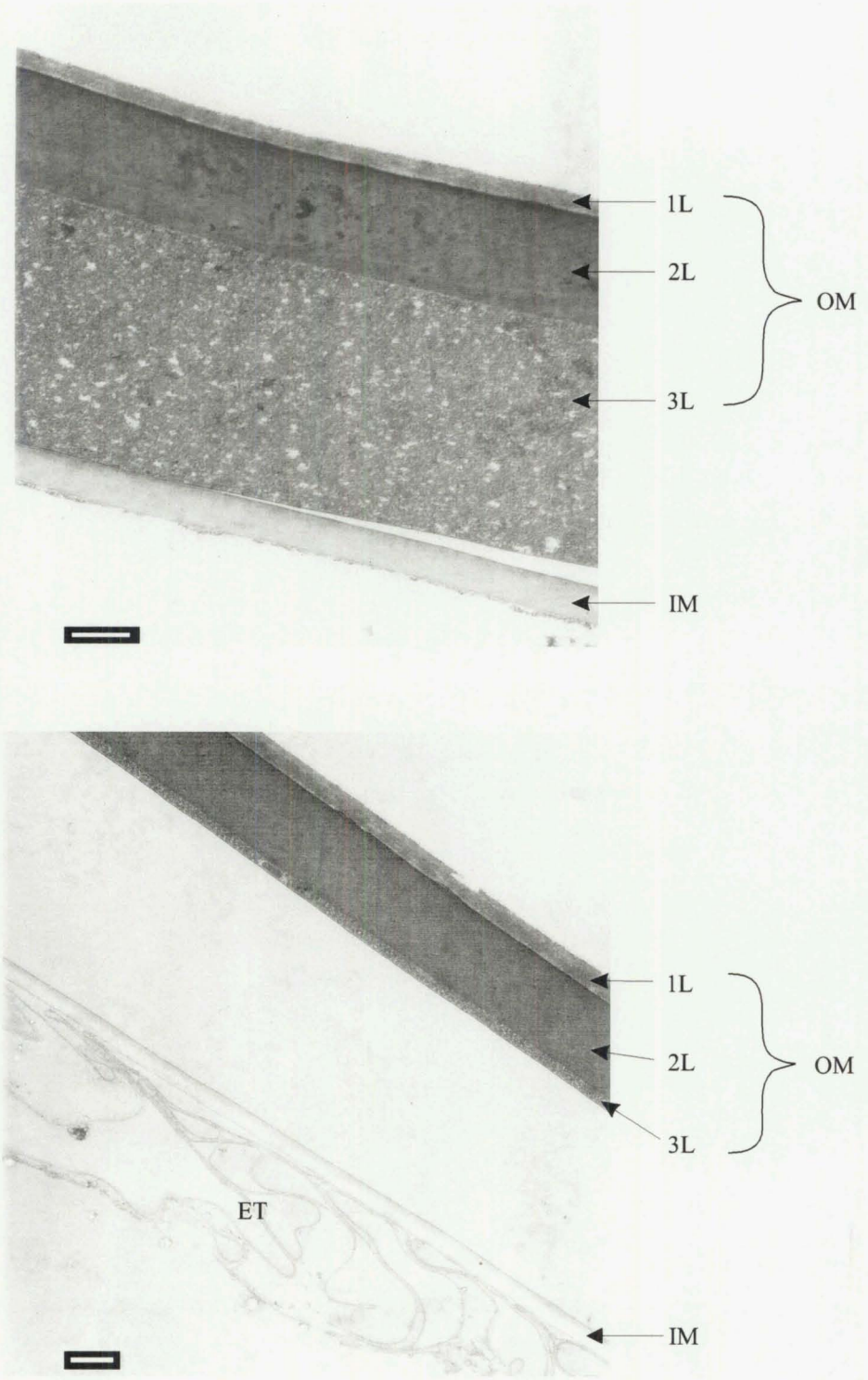



**Figure 2.13** Transmission electron microscope micrographs showing egg membranes and tissue of the embryo at Stage 4 of development of *H. edwardsii.* 1L = Layer 1, 2L = Layer 2, 3L = Layer 3 of the outer membrane, ET = embryonic tissue, IM = inner membrane, OM = outer membrane. Scale bars = 500 nm.

## DISCUSSION

The sequence of events observed during the embryonic development of these two crab species, *H. edwardsii* and *H. crenulatus*, agreed in general with that of other species (Amsler & George, 1984; Bas & Spivak, 2000; Helluy & Beltz, 1991; Jones & Simons, 1982; Leelapiyanart, 1996; Pandian, 1970; Wear, 1974). Different authors have recognized different stages in the development of schemes for staging embryonic development of brachyurans. In the present study, I defined five main developmental stages for eggs of both *H. edwardsii* and *H. crenulatus*. The criteria for characterizing these stages were based on cleaving of yolk, formation of blastula, gastrulation, yolk depletion/division, eye development, heart beat and the relative size of the egg. Total egg volume increased with development as for other crab eggs (Wear, 1974). For both species, egg volumes were relatively constant at early development (stage 1) and slightly increased after gastrulation. There was about a 1.5 fold increase in the total egg volume of both species, between spawning and hatching (Tables 2.1 & 2.2).

The rate of development of eggs of *H. edwardsii* was slower than *H. crenulatus* and this may perhaps be related to the size of the egg as noted by Wear (1974). At constant temperature, 15 °C, the average incubation period recorded for *H. edwardsii* was 62.0 ± 3.1 days. Literature suggests that eggs were incubated for about 42 days and female had only a single batch of eggs each year (review McLay, 1988). This discrepancy of incubation times may due to the differences in the incubation temperatures as suggested by Wear (1974). From my field observations at Waipara, I found that *H. edwardsii* started spawning around mid April and about 90% of crabs carried blastula stage eggs by the end of that month. Eggs at the last stage were found mostly in June and July. These data are therefore consistent with a single brood cycle for this species.

According to Clark (1987), the eggs of *H. crenulatus* in the Avon-Heathcote estuary are incubated by females for 8 - 12 weeks. In the present study the incubation time was about 6 weeks (43.0 ± 1.8 days) at 15 °C. Again, this may be due to the temperature difference, or perhaps other environmental parameters, which affect the

rate of development. I observed that ovigerous females occurred from August to late January at my field sites (Avon-Heathcote estuary) and this observation is consistent with others (review McLay, 1988). These observations simply support the suggestion that these crabs lay more than one batch of eggs per season. I experienced difficulties collecting ovigerous crabs carrying stage 1 eggs during my study period. This may be related to the behavioural adaptations of crabs carrying newly spawned eggs to avoid harsh environmental changes that may affect the early stage embryos. This suggestion is supported by the findings discussed in Chapter 3 that eggs in the early stages of development are less tolerant to salinity changes than later stages. However, the behaviour of crabs was not investigated in this study.

Light microscopical observations and ultrastructural studies of these eggs showed the presence of two distinct egg membranes (i.e. inner and outer membranes), which is consistent with the findings for other brachyurans (Charmantier & Aiken, 1987; Cheung, 1966; El-Sherief, 1993; Goudeau & Lachaise, 1983; Leelapiyanart, 1996; Young, 1937). It was found that the outer egg membrane was thick and tough whereas the inner membrane was thin in these eggs. Similar observations in the egg membranes was reported by El-Sherief (1993) that the egg of *Portunus pelagicus* is protected by an inner chorion (= inner membrane) and middle chitinous membrane (= inner layer of the outer membrane). He further commented from the evidence gathered from histological studies that the chitinous membrane is tanned and calcified.

In the present study, the outer egg membrane was found to consist of 3 layers which presumably correspond to the "trichromatic" membrane described by Cheung (1966). TEM observations suggest that the funiculus (or stalk) for attachment is formed of the outermost layer of the trichromatic membrane in these eggs as seen in other species (Goudeau & Lachaise, 1983). The thickness of these 3 layers of the outer membrane was variable at different positions (Figure 12). It is unclear whether changes in thickness also occurred with development.

Charmantier & Aiken (1987) reported that the inner egg membrane of late stage eggs of *Homarus americanus* consists of two layers. One is an embryonic cuticle, while the other-produced at the nauplius stage- is a cuticle resulting from an ecdysis. A

similar observation was recorded in the eggs of *Heterozius rotundifrons* (Leelapiyanart, 1996) and *Portunus pelagicus* (El-Sherief, 1993). However, in the present study, there seemed to be no clear identifiable separation of layers in the inner membrane (TEM observations were made on postgastrula stages) and it was seen as a single layer (Figure 2.12).

Silver nitrate staining of the eggs of both *H. edwardsii* and *H. crenulatus* revealed a dark-staining area associated with the egg membranes. Except for stage 1 eggs, the silver staining patch was located over the major mass of yolk at the opposite pole of the egg to the germinal disk or the embryo proper. In cleavage stages, silver staining was more diffuse and patchy. A transition stage showing both a dark staining area and diffuse patches were also observed in the early gastrula stage eggs (Figure 2.10). When late stage 5 eggs were stained with $AgNO_3$, it was observed that the patch was removed with the membranes on hatching and there was no sign of staining on the newly hatched larvae. However, it is not known whether this is due to the impermeability of the inner membrane to silver nitrate/or developer as suggested by Surbida (2001) for the eggs of *Armadillidium vulgare* (Isopoda). He reported that once the chorion is shed, the exposed vitelline membrane of early stage eggs stains rapidly. In the present study, it was impossible to localize a specified area on the hatched larvae which had been stained with silver nitrate. Although I have not made critical observations on the underlying tissue morphology of this patchy area, it can be said that the shape and the size of the patch were generally consistent for all developmental stages (except for stage 1 eggs).

The composition of the deposition observed on the egg surface in the present study was investigated with energy dispersive x-ray analysis method and was confirmed as $Ag^+$ and $Cl^-$. As the eggs were first washed in distilled water, this suggests that the stained area is a site where chloride ions are diffusing out of the embryo. Similarly, it has been demonstrated by X-ray microanalysis that the initial precipitate formed in the gills of the crab *Eriocheir sinensis* when stained with silver nitrate is mostly composed of AgCl (Barra, *et al.*, 1983), indicating that chloride ions released from the tissue were captured as silver chloride precipitates on the tissue surfaces. This AgCl deposition was formed between the outer and inner membranes of the egg from which it can be concluded that, in this region at least, the inner membrane is

permeable to $Cl^-$ ions and the outer membrane to $Ag^+$ ions. It is possible that in pregastrula stages, $Cl^-$ ions diffuse out at multiple locations between the blastomeres, resulting in their patchy staining.

The silver staining method has been used to localize the ion-transporting tissue areas (i.e. sites of presumed active uptake or excretion of salts) on the body surfaces in a variety of crustaceans (Barra, *et al.*, 1983; Dickson & Dillaman, 1991; Kikuchi & Matsumasa, 1993; Lindhjem *et al.*, 2000; McLusky, 1968: Talbot *et al.*, 1972). It is therefore, tentatively suggested that the silver staining patch may be a specialised epithelial structure that corresponds to the embryonic "dorsal organ" which has been described in many crustaceans which has a characteristic location & external morphology (Conte *et al.*, 1972, Ewing *et al.*, 1974; Fioroni, 1980; Hootman *et al.*, 1972; Hootman & Conte, 1975; Martin & Laverack, 1992; Meschenmoser, 1989; Morrit & Spicer, 1995).

On this basis it may be speculated that this could be the presumptive osmoregulatory epithelium in these eggs. Alternatively, the output of $Cl^-$ ions at this site might more logically be interpreted as a site of salt (and water) excretion, which is also a requirement for a hyper-osmoregulator.

# CHAPTER THREE

# OSMOREGULATION OF CRAB EMBRYOS

*Summary*

- Postgastrula stage embryos of both species were more tolerant of salinity change than cleavage stages and survived periods of dilution for many hours, even in 1% seawater.
- Pregastrula stage embryos were nearly isosmotic in all salinities whereas all postgastrula stages hyperosmoregulated.
- Gastrulation marks the critical stage for these embryos where transition from osmoconforming to hyperosmotic regulation commences.
- Osmoregulatory capacity was reduced in stage 5 embryos compared with stages 2-4 in both species.

## INTRODUCTION

It has been well documented that the ability to adapt to the variations in the environment enables a species to establish successfully in a given habitat. Theoretically, organisms can react in two ways when they confronted with salinity variations in their environment, if they are to survive: behaviourally (avoiding the problem) or by physiological adjustments. The life cycle of decapods includes an embryonic stage, which is often brooded on female pleopods. Therefore, embryos, in turn are always exposed to the same variations of conditions in the external habitat as the parent ovigerous females. However, the options available to developing embryos carried by ovigerous female crabs are more restricted. Avoidance of external salinity change would require behavioural response on the part of the parent crab. Thus there is an obvious advantage to the early development of salinity tolerance by embryos of euryhaline crab species.

The physiological mechanisms involved in response to osmotic stress can be grouped into two major patterns (Prosser, 1973). Firstly, animals may be osmotically labile (dependent) allowing their body-fluid concentrations to change with the medium; these are osmoconformers. Alternatively, animals may be osmotically stable (independent). When the medium changes, their internal concentration remains

relatively constant; these are osmoregulators. Many gradations exist between the extremes of lability and constancy. Also, an animal may conform to the medium osmotically in one concentration range and regulate in another.

In decapod crustaceans, the effect of salinity on embryonic development has been investigated in several species (Bas & Spivak, 2000) but information on the embryonic ontogeny of osmoregulation is scarce. Early appearance of strong regulatory capabilities in certain crustaceans, particularly in dilute media has been demonstrated by Anger & Charmantier (2000), Bas & Spivak (2000), Bouaricha *et al.* (1994), Charmantier *et al.* (1988, 1991, 2001), Felder *et al.* (1986), Haond *et al.* (1999), Jones & Simons (1982), Leelapiyanart (1996) and Susanto & Charmantier (2000, 2001). In the thalassinid ghost shrimp *Callianassa jamaicense*, hyper-osmotic ability is present at the time of hatching (Felder *et al.*, 1986). Ability to hyper-osmoregulate is existent in juveniles at hatch of crayfish *Astacus leptodactylus* (Susanto & Charmantier, 2000). The ontogeny of osmoregulation in embryos has also been studied in the homarid lobster *Homarus americanus* and found that larvae are osmoconformers before the acquisition of the ability to osmoregulate at metamorphosis (Charmantier & Aiken, 1987).

In a review Charmantier *et al.* (2001) distinguished three patterns of ontogeny of postembryonic osmoregulation in crustaceans from the results available in several species since the 1960s. In one group of species (pattern 1), osmoregulation varies little with developmental stage and the adults are often weak regulators/ stenohaline or osmoconformers. In another group (pattern 2), the adult type of osmoregulation is established in the first postembryonic stage; adults are euryhaline and generally live in environments where salinity is low, high or variable; this group includes freshwater species. In the third group of species (pattern 3), larvae osmoconform or are slightly able to osmoregulate; metamorphosis marks the appearance of the adult type of osmoregulation (massive tissue reorganization occurs at this time); Adults are mesohaline or euryhaline and live in environments of more or less variable salinity. Therefore, according to above classification, the ability to osmoregulate may occur at different stages of development depending on the pattern of ontogeny of osmoregulation.

The osmotic protection offered by the egg envelope during the period of embryonic development has been demonstrated in the embryos of *H. americanus* (Charmantier & Aiken, 1987) and *Astacus leptodactylus* (Susanto & Charmantier, 2001). They have shown that artificially opened embryos of these species transferred to dilute media were unable to osmoregulate. However, those protected by egg membranes remained hyper-osmotic at low salinity.

Therefore, from the available examples reviewed by Charmantier *et al* (2001), it has been hypothesised that developing embryos of decapods are osmoconformers or weak regulators and osmotic protection is provided by the egg envelopes until they acquire the adult type of osmoregulation. The mechanisms underlying this osmotic protection are still controversial. One hypothesis is based on the variable degree of permeability of the egg envelopes, which would be highly impermeable during most or part of development, preventing water invasion and ion loss at low salinities (Charmantier & Aiken, 1987; Krogh, 1939; Susanto & Charmantier, 2001; Valdes *et al.*, 1991). It has been reported that the permeability of egg membrane tends to increase towards the end of embryogenesis, perhaps following the action of proteolytic enzymes (De Vries & Forward, 1991). However, these mechanisms of osmoprotection affected by the egg envelopes are still hypothetical and should be further studied (Charmantier, 2001).

The New Zealand "purple rock crab", *Hemigrapsus edwardsii* (Hilgendorf, 1882) and "hairy-handed crab", *Hemigrapsus crenulatus* (H. Milne Edwards, 1837) are two intertidal crabs found along the coastline of New Zealand. There have been many studies on the ecology and physiology of these two adult crab species.

*H. edwardsii* is endemic to New Zealand and is much more abundant on southern shores. The habitat of *H. edwardsii* has been described as sheltered rocky, stony shores and stable protected in boulder beaches (Bedford & Leader, 1978). Generally, it occupies both the upper and middle shore levels and it has been found that in winter the crabs move further down the shore to occupy the area around low water level (Williams, 1969). The combined effects of temperature (5-25 $^{o}$C) and salinity (0-45 ppt) on survival of *H. edwardsii* have been investigated by Hicks (1973) and he found that for both summer and winter crabs, the dominant factor determining survival was salinity and confirmed that it is very euryhaline.

Phillips (1968) showed that the haemolymph of *H. edwardsii* is hyperosmotic to the external medium in dilute seawater but hyposmotic in more concentrated media. The osmoregulatory abilities of adult *H. edwardsii* have been reported by Bedford and Leader (1977). Although very euryhaline it possesses only a moderate capacity for regulation of the composition of its haemolymph in the face of large changes in external concentration. Later, Bedford and Leader (1978) found that *H. edwardsii* comes into osmotic and ionic balance with a new medium within 36 h, suggesting that the crabs are very permeable to water and ions.

In contrast to *H. edwardsii*, *H. crenulatus* seems to occupy a wide variety of habitats: under stones, burrowing in sand, mud, clay or earth, in sheltered marine or estuarine habitats and it is also recorded in sheltered places often in proximity to fresh water (Jones, 1976). According to Morton and Miller (1968), and from my personal observations, *H. crenulatus* is found in a similar environment to *H. edwardsii*, but more common in the places where the ground is muddy. The occurrence of *H. crenulatus* in such various habitats has lead to a great deal of research on its adaptations to fluctuating salinity. Its osmotic responses to altered salinity appear to be similar to those of *H. edwardsii* in that it comes rapidly into balance (36-48 h) with a changed medium, and does not survive long periods of immersion in salinities lower than 20% seawater (Jackson, 1976; Taylor, H.H., personal communication). Hicks (1973) investigated the combined effects of temperature and salinity on *H. crenulatus* and found that this species is well adapted to the salinity and temperature variations which occur in its habitat and suggested that burrowing may enable this crab species to avoid the most detrimental combinations. Jones (1976) and Bloomfield (1982) also confirmed that *H. crenulatus* is euryhaline crab.

As for other decapods, *H. edwardsii* and *H. crenulatus* brood their embryos externally attached to the pleopods of the female crab. Therefore, the developing embryos must be exposed to the variations in salinity in the environment due to rain, freshwater runoff and tidal effects throughout development from single cell stage to hatching larvae. Evidence has been documented above that the adult crabs of *H. edwardsii* and *H. crenulatus* are capable of surviving in wide ranges of salinity, both spatially and temporally and that they are hyperosmotic regulators in dilute seawater. Little is known about the embryos and the development of embryos of these two species in

such changeable habitats. Successful brood development of *H. crenulatus* between 18-36 ppt salinity with development time increasing with decreasing salinity was reported by Clark (1987). Clearly if the embryos were more susceptible to dilution than the adults, this would place a restriction on the movement of brooding females. It is thus of interest to investigate the salinity tolerance of developing embryos and the physiological basis of such tolerance.

Therefore, the main objectives of this chapter were to examine the tolerance of embryos of *H. edwardsii* and *H. crenulatus* at different developmental stages to short-term changes in salinity and also to study the effect of prolonged salinity change on their development. It was also of interest to know whether these embryos can osmoregulate and the osmoregulatory capabilities at different developmental stages. The putative osmoregulatory epithelium demonstrated in Chapter 2 suggests that an osmoregulatory capability might already exist in these embryos at an early stage. Two main protocols were used to differentiate between short-term (acute) and long-term (chronic) effects of osmotic stress; i.e. embryos at different developmental stages were exposed to different salinities for short periods (6, 24 and 96 h) and ovigerous crabs were reared at different salinities from spawning to hatching.

It is shown that the embryos are remarkably tolerant of periods of dilution of many hours, that they hyper-osmoregulate in hyposaline waters throughout their development, and that gastrulation marks the critical stage when the capacity to osmoregulate commences.

## MATERIALS AND METHODS

### Maintenance of ovigerous crabs

Ovigerous female *Hemigrapsus edwardsii* and of *H. crenulatus* were collected from the intertidal zones at Waipara and at Avon-Heathcote Estuary during the spawning period from mid April to late July and from August to January, respectively. The crabs were transported to the Department of Zoology, University of Canterbury and were maintained in an aquarium at 15 °C with recirculated, aerated 100% seawater (1000 mOsmol.kg$^{-1}$).

### Experimental media

Dilutions of seawater were used as test media for both salinity tolerance tests and osmoregulation studies. These test media were prepared using "Instant Ocean" salt (Aquarium Systems, Inc.) and tap water. Salinities were adjusted using a Wescor 5100 vapour pressure osmometer which had been calibrated with standard solutions of 100, 290 and 1000 mOsmol.kg$^{-1}$. The salinity of the control medium (i.e. 100% seawater) was 35.7‰ and the osmolality was 1050 mOsmol.kg$^{-1}$.

### Experimental design

Two main protocols were carried out to study the effect of salinity on survival and development and the hatching success of the embryos of both species; "acute" and "long term".

In acute experiments embryos were detached from the pleopods of ovigerous crabs taken from the aquarium at different developmental stages and exposed to a range of experimental salinity (1% to 100% seawater) for times ranging from 6 to 96 h.

In the long term experiments, ovigerous crabs with Stage 1 or Stage 2 embryos were reared at 15 °C in either 100%, 50% or 25% seawater until the embryos hatched.

In both protocols, salinity tolerance, total egg volume and osmolality of embryos were measured after the exposure time and at intervals. In long term experiments, viability of embryos with respect to development and hatching success was also observed.

The developmental stages and duration of development in each stage for the embryos of both *H. edwardsii* and *H. crenulatus* were distinguished for this study according to the criteria described in Chapter 2.

## Acute experiments

### (a) Salinity tolerance test

The detached embryos were conditioned in 100% seawater (1050 mOsmol.kg$^{-1}$) at 15 $^{o}$C for 24 h before confirming the viability of the detached embryos microscopically. They were then separated into groups of 25 and transferred directly to petri-dishes containing about 10 ml of seawater at different salinities (1%, 10%, 50% and 100% seawater) for the exposure time periods of 6, 24 and 96 h. Ten replicates of groups of 25 embryos at five different developmental stages were performed for each salinity and time. Counts of mortality were taken after the exposure time. At the end of the trial, the embryos were transferred back to 100% seawater in separate containers. The number of embryos out of 25 that survived after the treatments were recorded and observations of viabilities were repeated at 1 day and 7 day. The criteria for mortality were abnormal swelling, vacuolation in the embryos and lack of movement of the heart. Embryos were determined as being viable if they recovered their volume and appeared cytologically normal 7 days after return to normal seawater. Salinity tolerances are expressed as percentage survival.

### (b) Measurements of total volumes, solvent volumes and osmolalities of crab embryos in ranges of salinities and time

The total volumes of single embryos were calculated from the average diameters of 25 embryos using the ellipsoid formula; $V= \pi.D.d^2/6$ where "D" and "d" are

the greatest and smallest diameters respectively. Five replicates of 25 embryos at five different developmental stages were exposed to different salinities (1,10,50 and 100% seawater) for 6, 24 and 96 h. Diameters of treated embryos were measured by using an eyepiece micrometer after the exposure times and the total volume per egg was calculated.

To measure the osmolality of whole embryos, embryos were homogenized[1] in a small Eppendorf tube using a pestle attached to a motor. About 0.2 g of detached embryos at different stages were exposed to the experimental media. After the exposure times, the osmolality of the homogenized embryos was measured immediately using a Wescor 5100 vapour pressure osmometer. Five replicates were done for each stage, salinity and time.

For measurement of the solvent volume, osmolality measurements were repeated after diluting 30 µl of the homogenate of the whole embryos with 30 µl of distilled water. This diluted homogenate was mixed well so that debris was uniformly distributed. Osmolalities of diluted embryos were measured as above.

Solvent volume of one egg (v) was calculated from the osmolalities of diluted embryos according to the equation: $v = V.e/E$ where V (solvent volume of homogenate) = $d.w/(p-d)$

d = osmolality of diluted embryos
w = volume of water added
p = whole egg osmolality
e = volume of one egg
E = initial total volume of the homogenate

Osmolalities less than 200 $mOsmol.kg^{-1}$ were considered unreliable and were discarded. Thus, solvent volumes were not obtained for embryos in the lowest salinity treatments.

[1] See Chapter 8

**Long term exposure to different salinities**

Rearing of ovigerous crabs for this set of experiments was done in a recirculating tidal tank system (Leelapiyanart, 1996) (alternate periods of 6h 12 min immersion (high tide) and emersion (low tide) in a controlled temperature room (15 °C) under a 12h light and 12h dark cycles (see Appendix A). These conditions permitted the experiments to run with embryos *in situ*, i.e. the brood was not removed from the female.

Female ovigerous crabs collected from the field were numbered for identification and divided into 2 groups according to the developmental stage of the embryos (cleavage to blastula = Stage 1 or gastrula = Stage 2 – see Chapter 2) and placed into the three tidal system tanks at different salinities (100%, 50% and 25% seawater) after taking initial measurements (day 0) for total volume of embryos and osmolality. Ten female crabs for each group were maintained until the embryos hatched.

The osmolality of water was checked periodically and adjusted to the required level (either 1050 ± 5 mOsmol.kg $^{-1}$ , 500 ± 5 mOsmol.kg $^{-1}$ or 250 ± 5 mOsmol.kg $^{-1}$) during the course of the experiment. Water was changed completely every fortnight and crabs were fed with mussels.

Periodically, sampled embryos were removed to determine the viability and stage of development, total volume of single egg and the osmolality of whole embryos and diluted embryos. Methods were as described above.

**Statistical Analysis**

Osmolalities, total volumes and solvent volumes of embryos were expressed as mean ± S.E.M.. Salinity tolerances of embryos are expressed as percentage survival and these were transformed to angles using the arcsine square root transformation for statistical analysis. Differences of survival at different salinities were tested by one-way ANOVA, for each developmental stage. One way ANOVA was also used to test for the effect of salinity on the total egg volume for each stages. Salinity was the

categorical predictor (factor) in each case. Subsequent multiple comparisons of means were performed using Tukey HSD post-hoc test. Differences are reported as statistically significant when $P < 0.05$. The programme STATISTICA 6 was used for the statistical analyses.

# RESULTS

## Salinity tolerance in embryos of *H. edwar*dsii

A substantial proportion of both early and late stage embryos survived for periods of 6 to 96 h in seawater diluted to 50%, 10% and 1% (Figure 3.1). More than 75% of embryos at all developmental stages survived 96 h immersion in 50% seawater. However salinity was a highly significant factor affecting survival in all stages (see ANOVA table below). Late stage embryos were more tolerant and survived in lower salinities than did early stage embryos (Figure 3.1, Tukey post hoc tests). Mortality rates were high for stage 1 embryos and increased with exposure time and dilution. High survival rates were observed from stage 2 to stage 5 embryos in low salinities even after 96 h.

Results of one way ANOVA, testing the effect of the factor salinity on survival (arcsine transformed) for *H. edwardsii* embryos at each of 5 stages and three exposure times are shown in the table below.

| Stage | Exposure time (h) | F statistic ($F_{(1), 3, 36}$) | *P* |
|---|---|---|---|
| 1 | 6 | 361.8 | 0.00 |
| | 24 | 487.1 | 0.00 |
| | 96 | 580 | 0.00 |
| 2 | 6 | 9 | 0.0001 |
| | 24 | 12.9 | 0.000007 |
| | 96 | 22.4 | 0.000000 |
| 3 | 6 | 2.25 | 0.09 |
| | 24 | 18.7 | 0.000000 |
| | 96 | 100.8 | 0.00 |
| 4 | 6 | 1.0 | 0.4 |
| | 24 | 36.8 | 0.000000 |
| | 96 | 102.5 | 0.00 |
| 5 | 6 | 3.5 | 0.02 |
| | 24 | 18.7 | 0.000000 |
| | 96 | 283.8 | 0.00 |

Results obtained from the Tukey HSD post-hoc test are indicated in the Figure 3.1.

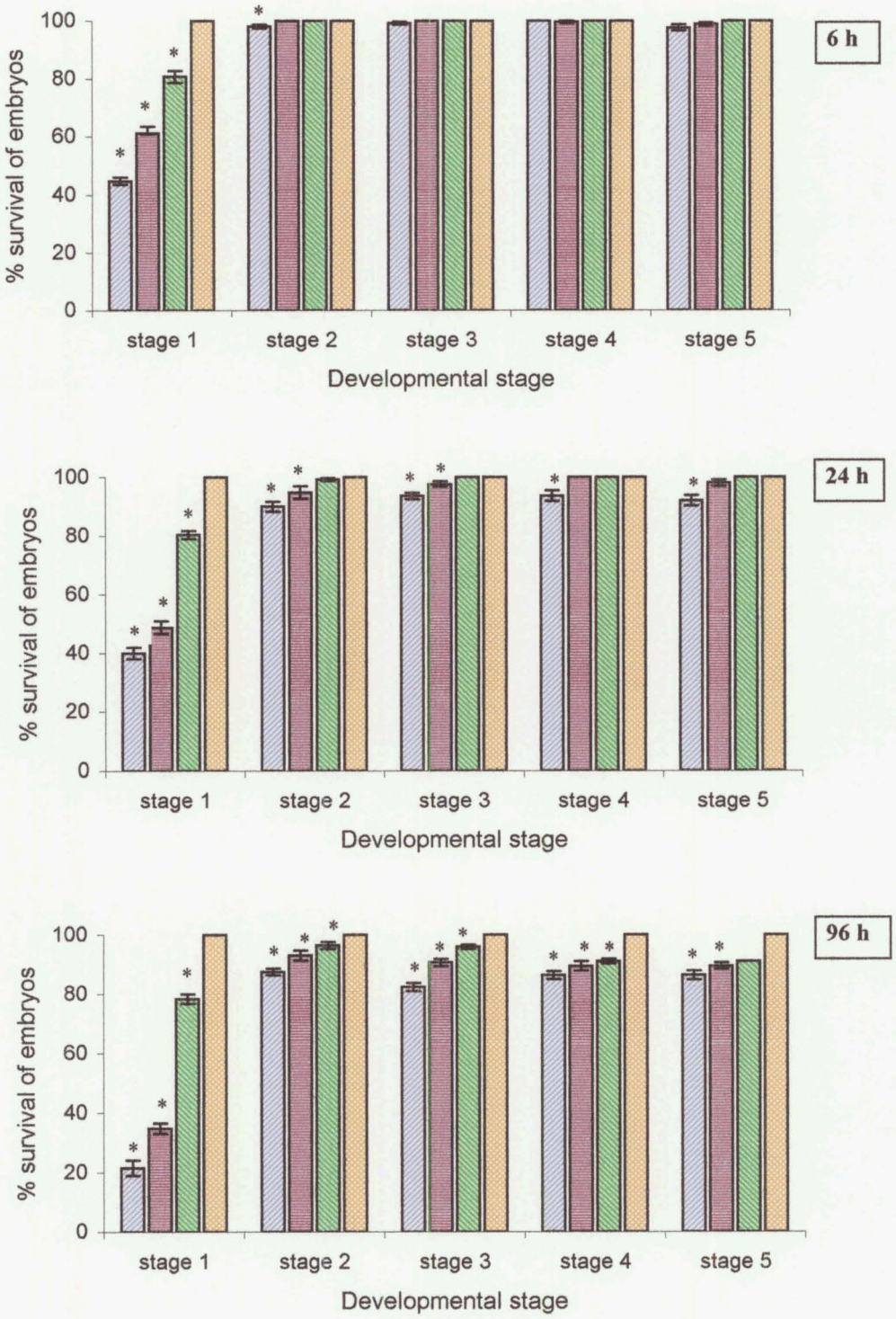



**Figure 3.1** Percentage survival of embryos at different stages of development in *Hemigrapsus edwardsii* after exposure to different salinities ( 100%, 50%, 10% and 1% seawater) for 6, 24 and 96 h at 15 °C. N = 10 replicates of 25 embryos for each stage and salinity. Values are means ± S.E.M. * significantly different from 100% seawater ($P < 0.05$).

**Salinity tolerance in embryos of *H. crenulatus***

As for *H. edwardsii*, salinity was a significant factor contributing to the survival of all stages of *H. crenulatus* embryos (ANOVA table below). Late stage embryos of *H. crenulatus* were more tolerant of dilution and survived lower concentrations of seawater than did early stage embryos (Figure 3.2). A similarly high mortality rate of stage 1 embryos in diluted seawater to that to that reported above for *H. edwardsii* was observed for *H. crenulatus*. Higher survival rates were observed from stage 2 (post gastrula) onwards. In 50% sea water more than 90% of all post-gastrula stage embryos survived for 96 hrs.

Results of one way ANOVA, testing the effect of the factor salinity on survival (arcsine transformed) for *H. crenulatus* embryos at each of 5 stages and three exposure times are shown in the table below.

| Stage | Exposure time (h) | F statistic ($F_{(1), 3, 36}$) | *P* |
|---|---|---|---|
| 1 | 6 | 498 | 0.00 |
| | 24 | 1722.9 | 0.00 |
| | 96 | 427.7 | 0.00 |
| 2 | 6 | 19 | 0.0001 |
| | 24 | 39.4 | 0.000000 |
| | 96 | 72.7 | 0.000000 |
| 3 | 6 | 199.5 | 0.00 |
| | 24 | 57.2 | 0.000000 |
| | 96 | 80.2 | 0.000000 |
| 4 | 6 | 22.8 | 0.000000 |
| | 24 | 61.6 | 0.000000 |
| | 96 | 81.7 | 0.00 |
| 5 | 6 | 23.9 | 0.000000 |
| | 24 | 72.5 | 0.000000 |
| | 96 | 242.6 | 0.00 |

Results obtained from the Tukey HSD post-hoc test are indicated in the Figure 3.2.

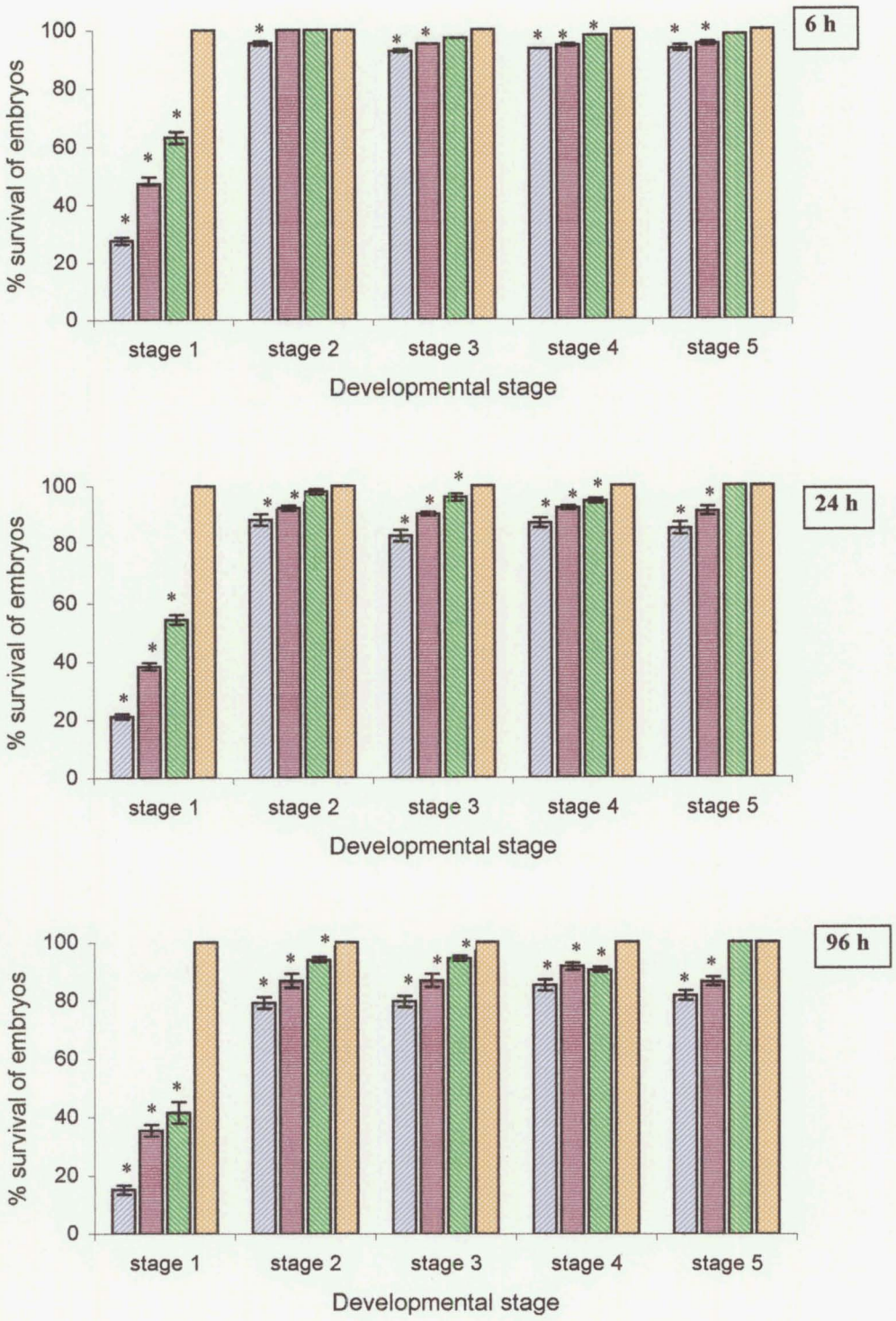



**Figure 3.2** Percentage survival of embryos at different stages of development in *Hemigrapsus crenulatus* after exposure to different salinities (100%, 50%, 10% and 1% seawater) for 6, 24 and 96 h at 15 °C. N = 10 replicates of 25 embryos for each stage and salinity. Values are means ± S.E.M.. * significantly different from 100% seawater ($P < 0.05$).

**Total volumes and solvent volumes of eggs of *H. edwardsii***

Tables 3.1 to 3.5 and Figure 3.3 present the mean values (± SE) of the total volume of eggs (nL) for different salinities after 6, 24 and 96 h exposure times from stage 1 to 5 respectively. There was about two-fold increase in the egg volume at stage 1 embryos after 6 h in 1% seawater. This was significantly different from 100% seawater at $P < 0.05$ (ANOVA results below, Tukey post hoc, Figure 3.3). The total volume of post gastrula stage embryos was not affected by the hyposaline exposures. Little volume change over the same range of salinity was recorded for stages 2 to 5 for 6, 24 and 96 h other than the increase of total volume of embryos that occurs with development in normal seawater.

Results of one way ANOVA, testing effect of the factor salinity on total volume of *H. edwardsii* embryos at each of 5 stages and three exposure times are shown in the table below.

| Stage | Exposure time (h) | F statistic ($F_{(1), 3, 16}$) | *P* |
|---|---|---|---|
| 1 | 6 | 225.4 | 0.000000 |
| | 24 | 169.2 | 0.000000 |
| | 96 | 243.8 | 0.000000 |
| 2 | 6 | 0.09 | 0.96 |
| | 24 | 0.08 | 0.96 |
| | 96 | 4.55 | 0.02 |
| 3 | 6 | 10.9 | 0.00037 |
| | 24 | 9.8 | 0.00066 |
| | 96 | 16.4 | 0.000038 |
| 4 | 6 | 0.39 | 0.76 |
| | 24 | 1.35 | 0.29 |
| | 96 | 2.68 | 0.08 |
| 5 | 6 | 0.6 | 0.63 |
| | 24 | 0.6 | 0.59 |
| | 96 | 3.18 | 0.05 |

Results obtained from the Tukey HSD post-hoc test are indicated in the Figure 3.3.

The calculated mean values (± SE) of the solvent volume per egg (nL) for all developmental stages in different salinities and for the periods of 6 to 96 h are presented in Tables 3.1 to 3.5. As the osmolality of diluted embryos in 10% and 1% seawater for stage 1 was not measured, the solvent volumes were not calculated.

**Table 3.1** Mean ± S.E.M. of Osmolality, Total volume & Solvent volume of embryos at Stage 1 of *Hemigrapsus edwardsii* after exposure to different salinities for 6, 24 and 96 h at 15 °C.

| Salinity of sea water (%) | Osmolality of sea water (mOsmol. $kg^{-1}$) | Osmolality of embryos (mOsmol. $kg^{-1}$) | | | Total Volume per egg (nL) | | | Solvent volume per egg (nL) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | after 6 h | after 24 h | after 96 h | after 6 h | after 24 h | after 96 h | after 6 h | after 24 h | after 96 h |
| 100% | 1050 | 1220 ± 15 | 1212 ± 19.4 | 1238 ± 4.92 | 20.4 ± 0.19 | 20.1 ± 0.6 | 20.5 ± 0.1 | 9.68 ± 0.3 | 10.12 ± 0.2 | 10.3 ± 0.4 |
| 50% | 525 | 646.7 ± 9.9 | 649 ± 32.2 | 599 ± 17.2 | 21.3 ± 0.43 | 22.4 ± 0.5 | 23.3 ± 0.3 | 14.2 ± 0.7 | 17.42 ± 1.1 | 18.6 ± 0.5 |
| 10% | 105 | 231.2 ± 11 | 230 ± 3.18 | 198 ± 8.98 | 43 ± 1.45 | 48.7 ± 0.2 | 52.5 ± 0.8 | | | |
| 1% | 10.5 | 152.5 ± 38 | 178 ± 17.3 | 108 ± 11.8 | 49.3 ± 1.24 | 48.2 ± 2.3 | 40 ± 1.7 | | | |

**Table 3.2** Mean ± S.E.M. of Osmolality, Total volume & Solvent volume of embryos at Stage 2 of *Hemigrapsus edwardsii* after exposure to different salinities for 6, 24 and 96 h at 15 °C.

| Salinity of sea water (%) | Osmolality of sea water (mOsmol. $kg^{-1}$) | Osmolality of embryos (mOsmol. $kg^{-1}$) | | | Total Volume per egg (nL) | | | Solvent volume per egg (nL) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | after 6 h | after 24 h | after 96 h | after 6 h | after 24 h | after 96 h | after 6 h | after 24 h | after 96 h |
| 100% | 1050 | 1254 ± 11 | 1269 ± 12.2 | 1237 ± 25.5 | 23.6 ± 0.57 | 23.9 ± 0.6 | 24.2 ± 0.3 | 11.4 ± 0.6 | 12 ± 0.4 | 13.3 ± 0.8 |
| 50% | 525 | 1128 ± 24 | 1168 ± 34.9 | 944 ± 18.7 | 23.8 ± 0.53 | 23.8 ± 0.5 | 24.4 ± 0.3 | 12.3 ± 0.9 | 12 ± 0.5 | 15.1 ± 0.6 |
| 10% | 105 | 1126 ± 21 | 1040 ± 44 | 771 ± 53 | 23.6 ± 0.49 | 23.9 ± 0.6 | 24.9 ± 0.3 | 12.4 ± 0.8 | 12.29 ± 0.9 | 18.7 ± 0.6 |
| 1% | 10.5 | 1072 ± 18 | 1005 ± 44.8 | 716 ± 53.8 | 24 ± 0.62 | 24.2 ± 0.6 | 25.6 ± 0.3 | 12.9 ± 0.7 | 13.4 ± 0.7 | 19.9 ± 0.8 |

**Table 3.3** Mean ± S.E.M. of Osmolality, Total volume & Solvent volume of embryos at Stage 3 of *Hemigrapsus edwardsii* after exposure to different salinities for 6, 24 and 96 h at 15 °C.

| Salinity of sea water (%) | Osmolality of sea water (mOsmol. $kg^{-1}$) | Osmolality of embryos (mOsmol. $kg^{-1}$) | | | Total Volume per egg (nL) | | | Solvent volume per egg (nL) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | after 6 h | after 24 h | after 96 h | after 6 h | after 24 h | after 96 h | after 6 h | after 24 h | after 96 h |
| 100% | 1050 | 1235 ± 8 | 1220 ± 18.7 | 1222 ± 3.22 | 28.8 ± 0.25 | 30 ± 0.5 | 29.6 ± 0.2 | 19.7 ± 0.4 | 20.89 ± 1.1 | 19.3 ± 0.3 |
| 50% | 525 | 1149 ± 9.8 | 1106 ± 16.7 | 942 ± 3.69 | 29 ± 0.11 | 30.9 ± 0.4 | 30.8 ± 0.2 | 20.2 ± 0.9 | 21.08 ± 0.8 | 22.6 ± 0.5 |
| 10% | 105 | 1037 ± 17 | 1016 ± 22.6 | 743 ± 6.39 | 29.2 ± 0.11 | 30.9 ± 0.1 | 30.7 ± 0.2 | 21.1 ± 0.9 | 22.85 ± 1.1 | 23 ± 0.4 |
| 1% | 10.5 | 1018 ± 13 | 953 ± 17.9 | 658 ± 5.03 | 31.6 ± 0.48 | 31.8 ± 0.2 | 32.1 ± 0.4 | 22.3 ± 0.8 | 22.93 ± 1 | 30.2 ± 0.3 |

**Table 3.4** Mean ± S.E.M. of Osmolality, Total volume & Solvent volume of embryos at Stage 4 of *Hemigrapsus edwardsii* after exposure to different salinities for 6, 24 and 96 h at 15 °C.

| Salinity of sea water (%) | Osmolality of sea water (mOsmol. $kg^{-1}$) | Osmolality of embryos (mOsmol. $kg^{-1}$) | | | Total Volume per egg (nL) | | | Solvent volume per egg (nL) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | after 6 h | after 24 h | after 96 h | after 6 h | after 24 h | after 96 h | after 6 h | after 24 h | after 96 h |
| 100% | 1050 | 1247 ± 10 | 1264 ± 9.34 | 1264 ± 4.3 | 32.2 ± 0.32 | 32.4 ± 0.2 | 32.8 ± 0.2 | 24.3 ± 0.3 | 25.33 ± 1.1 | 27.9 ± 0.8 |
| 50% | 525 | 1173 ± 3.2 | 1176 ± 17.2 | 1063 ± 14.8 | 32.3 ± 0.51 | 32.5 ± 0.1 | 35.1 ± 0.7 | 24 ± 0.3 | 26.29 ± 0.6 | 30.6 ± 0.9 |
| 10% | 105 | 1091 ± 6.8 | 1091 ± 9.58 | 750 ± 20.9 | 32.6 ± 0.29 | 32.3 ± 0.1 | 34.6 ± 1 | 25.8 ± 0.4 | 27.43 ± 1.6 | 31.3 ± 2 |
| 1% | 10.5 | 1094 ± 5.5 | 1069 ± 12.5 | 655 ± 17.9 | 32.7 ± 0.33 | 32.9 ± 0.4 | 34.4 ± 0.7 | 25.1 ± 0.4 | 29.57 ± 1.5 | 31.4 ± 1.3 |

**Table 3.5** Mean ± S.E.M. of Osmolality, Total volume & Solvent volume of embryos at Stage 5 of *Hemigrapsus edwardsii* after exposure to different salinities for 6, 24 and 96 h at 15 °C.

| Salinity of sea water (%) | Osmolality of sea water (mOsmol. $kg^{-1}$) | Osmolality of embryos (mOsmol. $kg^{-1}$) | | | Total Volume per egg (nL) | | | Solvent volume per egg (nL) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | after 6 h | after 24 h | after 96 h | after 6 h | after 24 h | after 96 h | after 6 h | after 24 h | after 96 h |
| 100% | 1050 | 1271 ± 5.8 | 1284 ± 33.8 | 1286 ± 7.62 | 36.3 ± 0.08 | 36.7 ± 0.3 | 37.3 ± 0.3 | 30.3 ± 0.2 | 33.37 ± 0.7 | 36.6 ± 1.1 |
| 50% | 525 | 1175 ± 1.9 | 1232 ± 34.8 | 1001 ± 20.5 | 36.5 ± 0.12 | 36.7 ± 0.4 | 37.5 ± 0.5 | 30.7 ± 0.8 | 34.37 ± 1 | 35.9 ± 1.2 |
| 10% | 105 | 1115 ± 16 | 1128 ± 12.4 | 554 ± 14.8 | 36.3 ± 0.26 | 36.8 ± 0.4 | 38.3 ± 0.3 | 32 ± 0.5 | 34.44 ± 1.4 | 35.6 ± 1.8 |
| 1% | 10.5 | 1056 ± 8.6 | 1118 ± 17.9 | 459 ± 21.6 | 36.4 ± 0.07 | 37.3 ± 0.3 | 38.6 ± 0.4 | 35.1 ± 0.2 | 34.37 ± 1.6 | 34.2 ± 5 |

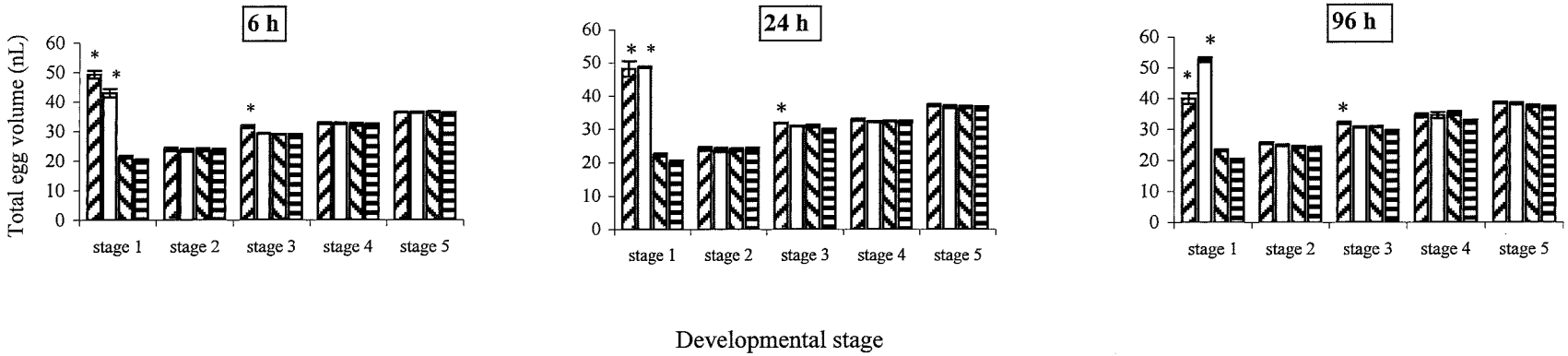



**Figure 3.3** Total volume of eggs at different salinities and different developmental stages of *Hemigrapsus edwardsii* after 6, 24 and 96 h at 15 °C. ( 1% seawater, 10% seawater, 50% seawater & 100% seawater). Values are mean ± S.E.M. of five replicates for each stage and salinity. * significantly different from 100% seawater ($P < 0.05$).

There was an increase in the solvent volumes with development of embryos and in dilution. It was found that about 50% of the total egg volume is solvent volume (i.e. free water) for early stages and about 90% for the late stages in normal embryos developed in 100% seawater.

**Total volumes and solvent volumes of embryos of *H. crenulatus***

As for *H. edwardsii*, the total volume of pre-gastrula stage embryos significantly increased in hyposaline water but was relatively unaffected by salinity in post-gastrula stage embryos. Tables 3.6 to 3.10 present the mean values ± S.E.M of the total volume per embryos (nL) from stage 1 to stage 5 respectively. A significant increase in the total volume of stage 1 embryos (pregastrula) was observed in 50%, 10% and 1% seawater ($p<0.05$) compared with that of 100% seawater (Fig. 3.4). Interestingly, the volume of stage 1 embryos decreased significantly again in the period of 24 to 96 h in 1% seawater (ANOVA results below, Tukey post hoc $P < 0.05$).

Results of one way ANOVA, testing effect of the factor salinity on total volume of *H. crenulatus* embryos at each of 5 stages and three exposure times are shown in the table below.

| Stage | Exposure time (h) | F statistic ($F_{(1), 3, 16}$) | *P* |
|---|---|---|---|
| 1 | 6 | 20.4 | 0.000010 |
| | 24 | 17.9 | 0.000023 |
| | 96 | 12.4 | 0.000190 |
| 2 | 6 | 2.4 | 0.101 |
| | 24 | 2.0 | 0.149 |
| | 96 | 4.4 | 0.0198 |
| 3 | 6 | 2.2 | 0.128 |
| | 24 | 20.2 | 0.000011 |
| | 96 | 16.0 | 0.000045 |
| 4 | 6 | 0.0 | 0.99 |
| | 24 | 3.3 | 0.04 |
| | 96 | 3.5 | 0.04 |
| 5 | 6 | 6.3 | 0.0049 |
| | 24 | 29.0 | 0.000001 |
| | 96 | 21.2 | 0.000008 |

Results obtained from the Tukey HSD post-hoc test are indicated in the Figure 3.4.

Changes in the calculated solvent volume per egg at different salinities are presented in Tables 3.6 to 3.10 from stage 1 to 5 respectively. As for *H. edwardsii*, the solvent volume of a single egg increased with development of embryos and with decreasing salinity. Similarly, the solvent volumes of early and late stage embryos were about 50% and 90% respectively of the total volume of embryos developing in normal seawater.

**Osmoregulation in a range of salinities for embryos of *H. edwardsii***

Embryos at all stages, except stage 1, were markedly hyperosmotic to the external medium. Stage 1 embryos were nearly isosmotic in all salinities from 6 to 96 h but consistently slightly above the theoretical isosmotic line (Figure 3.5). Tables 3.1 to 3.5 present the mean values ± S.E.M of the time course of changes in osmolalities from stages 1 to 5 in 100%, 50%, 10% and 1% sea water. It was found that osmolality of post gastrula stage embryos was relatively insensitive to the external salinity and maintained hyperosmotic condition during the exposure times. There is a general fall in osmolality of embryos after 96 h. This decrease in the osmolality was particularly observed in stage 5 embryos after 96 h in 1% seawater.

**Osmoregulation in a range of salinities for embryos of *H. crenulatus***

As in *H. edwardsii*, embryos at all stages were hyperosmotic to the external medium except stage 1 embryos (Figure 3.6). Stage 1 embryos osmoconformed in low salinities. Time course of changes in the osmolalities and the mean values (± SE) of the whole egg osmolality for all developmental stages are presented in Tables 3.6 to 3.10. As for *H. edwardsii*, osmolality of embryos decreased after 96 h. However, embryos at stages 2, 3 and 4 could maintain a considerably consistent hyperosmotic condition throughout the time period observed. In contrast to *H. edwardsii*, stage 5 embryos tended to move to isosmotic condition after 96 h in dilute seawater.

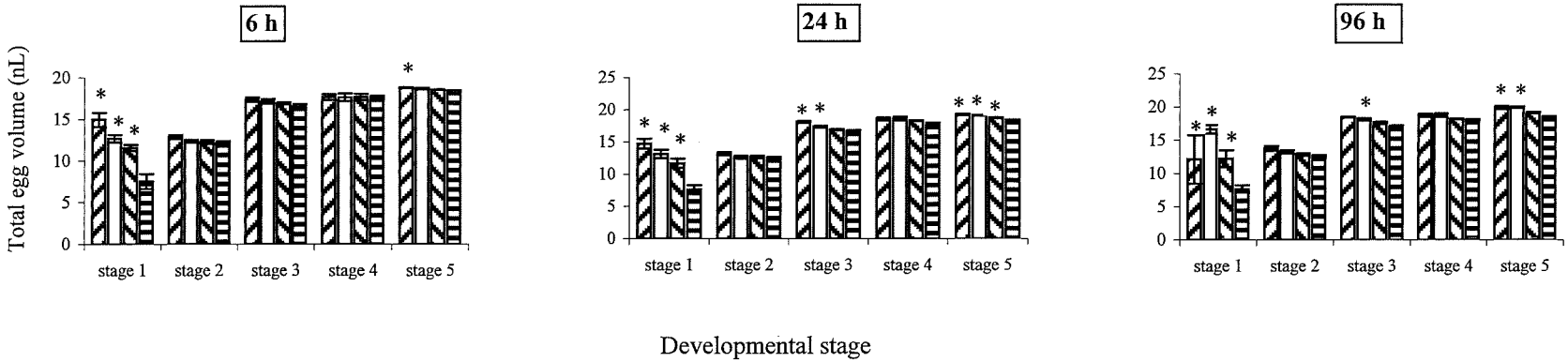



**Figure 3.4** Total volume of eggs at different salinities and different developmental stages of *Hemigrapsus crenulatus* after 6, 24 and 96 h at 15 °C. (▨ 1% seawater, □ 10% seawater, ▧ 50% seawater & ▤ 100% seawater). Values are mean ± S.E.M. of five replicates for each stage and salinity. * significantly different from 100% seawater ($P < 0.05$).

**Table 3.6** Mean ± S.E.M. of Osmolality, Total volume & Solvent volume of embryos at Stage 1 of *Hemigrapsus crenulatus* after exposure to different salinities for 6, 24 and 96 h at 15 °C.

| Salinity of sea water (%) | Osmolality of sea water (mOsmol. $kg^{-1}$) | Osmolality of embryos (mOsmol. $kg^{-1}$) | | | Total Volume per egg (nL) | | | Solvent volume per egg (nL) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | after 6 h | after 24 h | after 96 h | after 6 h | after 24 h | after 96 h | after 6 h | after 24 h | after 96 h |
| 100% | 1050 | 1206 ± 27 | 1210 ± 22.4 | 1207 ± 23.1 | 7.55 ± 0.91 | 7.63 ± 0.7 | 7.67 ± 0.5 | 3.67 ± 0.2 | 3.94 ± 0.5 | 3.96 ± 0.6 |
| 50% | 525 | 687 ± 54 | 680 ± 22.2 | 647 ± 24 | 11.5 ± 0.36 | 11.7 ± 0.7 | 12.2 ± 1.3 | 6.68 ± 1.5 | 8.45 ± 0.7 | 9.02 ± 0.8 |
| 10% | 105 | 224 ± 13 | 220 ± 5.18 | 187 ± 4.72 | 12.7 ± 0.44 | 13.1 ± 0.7 | 16.7 ± 0.6 | | | |
| 1% | 10.5 | 197 ± 12 | 166 ± 13.1 | 135 ± 8.23 | 15 ± 0.85 | 14.7 ± 0.8 | 12.1 ± 3.7 | | | |

**Table 3.7** Mean ± S.E.M. of Osmolality, Total volume & Solvent volume of embryos at Stage 2 of *Hemigrapsus crenulatus* after exposure to different salinities for 6, 24 and 96 h at 15 °C.

| Salinity of sea water (%) | Osmolality of sea water (mOsmol. $kg^{-1}$) | Osmolality of embryos (mOsmol. $kg^{-1}$) | | | Total Volume per egg (nL) | | | Solvent volume per egg (nL) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | after 6 h | after 24 h | after 96 h | after 6 h | after 24 h | after 96 h | after 6 h | after 24 h | after 96 h |
| 100% | 1050 | 1229 ± 20 | 1235 ± 15.9 | 1249 ± 11.6 | 12.2 ± 0.15 | 12.5 ± 0.2 | 12.6 ± 0.1 | 7.69 ± 0.2 | 8.06 ± 0.4 | 8.72 ± 0.3 |
| 50% | 525 | 1142 ± 5.6 | 1111 ± 32.5 | 946 ± 18.3 | 12.3 ± 0.22 | 12.7 ± 0.2 | 12.9 ± 0.2 | 8.18 ± 0.3 | 8.94 ± 0.4 | 10 ± 0.2 |
| 10% | 105 | 1041 ± 9.8 | 1039 ± 18.1 | 819 ± 23 | 12.4 ± 0.16 | 12.6 ± 0.2 | 13.3 ± 0.2 | 8.53 ± 0.5 | 8.73 ± 0.4 | 11.2 ± 0.5 |
| 1% | 10.5 | 1015 ± 23 | 1022 ± 15.3 | 740 ± 27 | 12.9 ± 0.24 | 13.2 ± 0.2 | 13.8 ± 0.4 | 8.62 ± 0.5 | 9.13 ± 0.4 | 12.1 ± 0.4 |

**Table 3.8** Mean ± S.E.M. of Osmolality, Total volume & Solvent volume of embryos at Stage 3 of *Hemigrapsus crenulatus* after exposure to different salinities for 6, 24 and 96 h at 15 °C.

| Salinity of sea water (%) | Osmolality of sea water (mOsmol. $kg^{-1}$) | Osmolality of embryos (mOsmol. $kg^{-1}$) | | | Total Volume per egg (nL) | | | Solvent volume per egg (nL) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | after 6 h | after 24 h | after 96 h | after 6 h | after 24 h | after 96 h | after 6 h | after 24 h | after 96 h |
| 100% | 1050 | 1251 ± 5.2 | 1258 ± 6.77 | 1245 ± 12.4 | 16.6 ± 0.26 | 16.6 ± 0.2 | 17 ± 0.2 | 13.7 ± 0.3 | 13.86 ± 0.3 | 14 ± 0.2 |
| 50% | 525 | 1179 ± 6.5 | 1138 ± 7.57 | 864 ± 3.42 | 16.9 ± 0.11 | 16.9 ± 0.1 | 17.6 ± 0.2 | 14 ± 0.2 | 14.25 ± 0.4 | 16.3 ± 0.2 |
| 10% | 105 | 1072 ± 26 | 1028 ± 10.9 | 717 ± 8.16 | 17.2 ± 0.26 | 17.4 ± 0.1 | 18.1 ± 0.2 | 13.1 ± 0.1 | 15.24 ± 0.6 | 16.5 ± 0.4 |
| 1% | 10.5 | 1058 ± 15 | 1002 ± 16 | 560 ± 7.39 | 17.4 ± 0.25 | 18.1 ± 0.1 | 18.5 ± 0.1 | 14.5 ± 0.9 | 15.98 ± 0.6 | 18.1 ± 0.1 |

**Table 3.9** Mean ± S.E.M. of Osmolality, Total volume & Solvent volume of embryos at Stage 4 of *Hemigrapsus crenulatus* after exposure to different salinities for 6, 24 and 96 h at 15 °C.

| Salinity of sea water (%) | Osmolality of sea water (mOsmol. $kg^{-1}$) | Osmolality of embryos (mOsmol. $kg^{-1}$) | | | Total Volume per egg (nL) | | | Solvent volume per egg (nL) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | after 6 h | after 24 h | after 96 h | after 6 h | after 24 h | after 96 h | after 6 h | after 24 h | after 96 h |
| 100% | 1050 | 1266 ± 13 | 1252 ± 5.65 | 1222 ± 10.1 | 17.6 ± 0.19 | 17.7 ± 0.3 | 18 ± 0.1 | 15.2 ± 0.4 | 15.27 ± 0.3 | 17.5 ± 0.4 |
| 50% | 525 | 1153 ± 7.3 | 1152 ± 10.9 | 967 ± 16.2 | 17.7 ± 0.36 | 18.3 ± 0 | 18.2 ± 0 | 15.8 ± 0.6 | 15.44 ± 0.5 | 17.7 ± 0.4 |
| 10% | 105 | 1116 ± 17 | 1048 ± 10.7 | 739 ± 45.7 | 17.7 ± 0.44 | 18.6 ± 0.3 | 18.8 ± 0.3 | 15.8 ± 0.4 | 15.99 ± 0.3 | 18 ± 0.5 |
| 1% | 10.5 | 1069 ± 5.3 | 1028 ± 12.9 | 651 ± 38.2 | 17.7 ± 0.35 | 18.6 ± 0.2 | 18.7 ± 0.2 | 16 ± 0.6 | 16.78 ± 0.5 | 18 ± 0.4 |

**Table 3.10** Mean ± S.E.M. of Osmolality, Total volume & Solvent volume of embryos at Stage 5 of *Hemigrapsus crenulatus* after exposure to different salinities for 6, 24 and 96 h at 15 °C.

| Salinity of sea water (%) | Osmolality of sea water (mOsmol. $kg^{-1}$) | Osmolality of embryos (mOsmol. $kg^{-1}$) | | | Total Volume per egg (nL) | | | Solvent volume per egg (nL) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | after 6 h | after 24 h | after 96 h | after 6 h | after 24 h | after 96 h | after 6 h | after 24 h | after 96 h |
| 100% | 1050 | 1279 ± 11 | 1283 ± 4.17 | 1197 ± 21.6 | 18.4 ± 0.04 | 18.4 ± 0 | 18.5 ± 0.1 | 17 ± 0.3 | 17.64 ± 0.6 | 17.3 ± 0.5 |
| 50% | 525 | 1170 ± 5.1 | 1102 ± 25.3 | 668 ± 22.6 | 18.6 ± 0.04 | 18.7 ± 0.1 | 19.1 ± 0.1 | 16.6 ± 0.9 | 17.98 ± 0.7 | 17.7 ± 0.9 |
| 10% | 105 | 1031 ± 7.1 | 836 ± 70.7 | 237 ± 6.16 | 18.7 ± 0.1 | 19.2 ± 0.1 | 20 ± 0.1 | 17.9 ± 0.5 | 18.28 ± 0.7 | 18.2 ± 0.5 |
| 1% | 10.5 | 983.8 ± 12 | 739 ± 80.6 | 135 ± 9.03 | 18.8 ± 0.05 | 19.3 ± 0.1 | 19.9 ± 0.2 | 18 ± 0.2 | 18.06 ± 0.4 | 18.9 ± 1.4 |

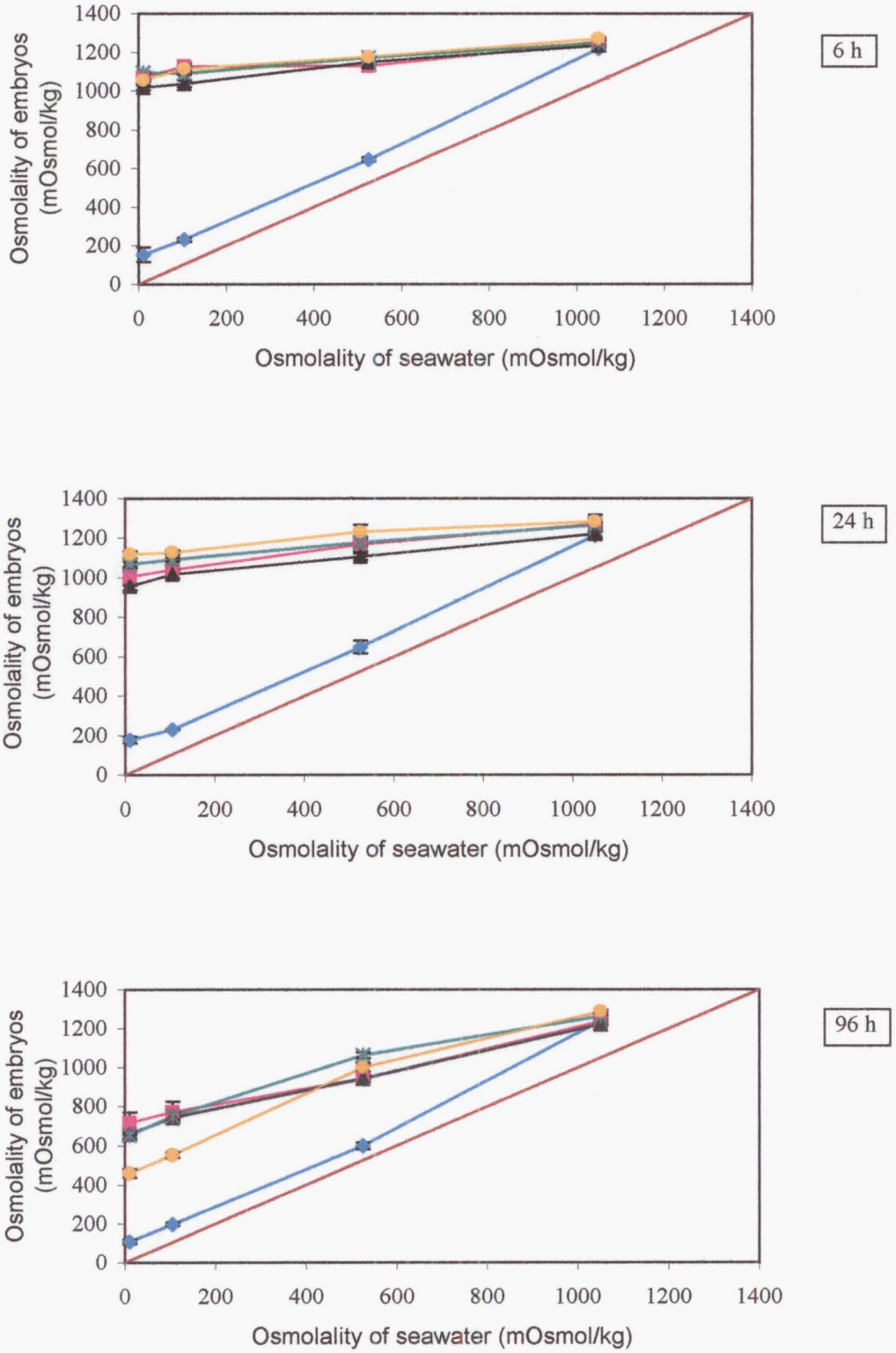



**Figure 3.5** Osmolality of embryos of *Hemigrapsus edwardsii* as a function of medium osmolality for different developmental stages ( stage 1, stage 2, stage 3, stage 4, stage 5 and isosmotic) after 6, 24 and 96 h at 15 °C. N = 5 replicates for each stage and salinity. Values are mean ± S.E.M.

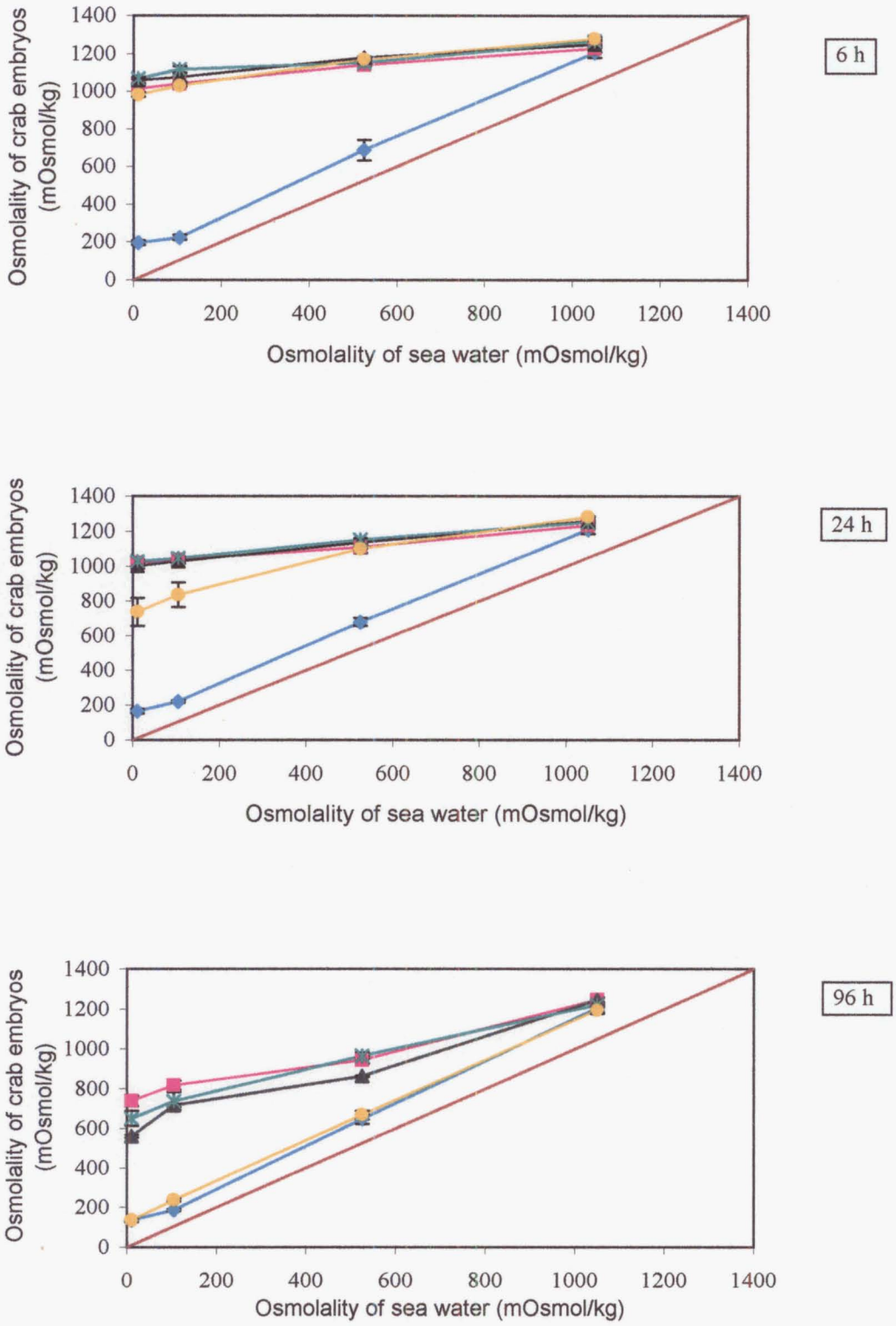



**Figure 3.6** Osmolality of embryos of *Hemigrapsus crenulatus* as a function of medium osmolality for different developmental stages ( stage 1, stage 2, stage 3, stage 4, stage 5 and isosmotic) after 6, 24 and 96 h at 15 °C. N = 5 replicates for each stage and salinity. Values are mean ± S.E.M.

**Development of embryos of *H. edwardsii* continuously exposed to low salinities**

The effect of continuous exposure to dilute seawater on the development of embryos was observed by introducing ovigerous crabs carrying embryos to the experimental tidal systems either at stage 1 or stage 2.

**(a) Viability and hatching success**

Figure 3.7 indicates the different developmental stages and the morphological changes of the embryos developed in three different salinities from stage 1 embryos, i.e. from Cleavage/Blastula stage to hatching. As expected the development was normal and they successfully hatched after 60 days in 100% seawater. In 50% seawater, development of embryos was generally similar to that of 100% seawater, but it was delayed and arrested at stage 5. They did not hatch successfully and about 10% produce immobile larvae after 70 days. In 25% seawater development of embryos didn't proceed beyond gastrula and the majority of ovigerous crabs aborted their embryos by day 30.

Commencing hyposaline exposure after gastrulation (in stage 2 embryos) markedly improved the survival of the embryos (Figure 3.8). Embryos were hatched successfully after about 50 days in 100% seawater. Although the development was slightly delayed, embryos were successfully hatched as active larvae in 50% seawater after about 60 days. The colour of the yolk cells of the embryos developed in 50% seawater was changed from yellow to white during the development and the larvae hatched contained much reduced yolk compared to the larvae hatched in 100% seawater (Figure 3.9). However, critical observations were not made on the rate of depletion of yolk in the embryos developed in low salinities with that of 100% seawater. In 25% seawater, development of embryos proceeded further than in stage 1 embryos but they were unable to develop and hatch successfully. Abortion of embryos was seen after about 60 days in 25% seawater.

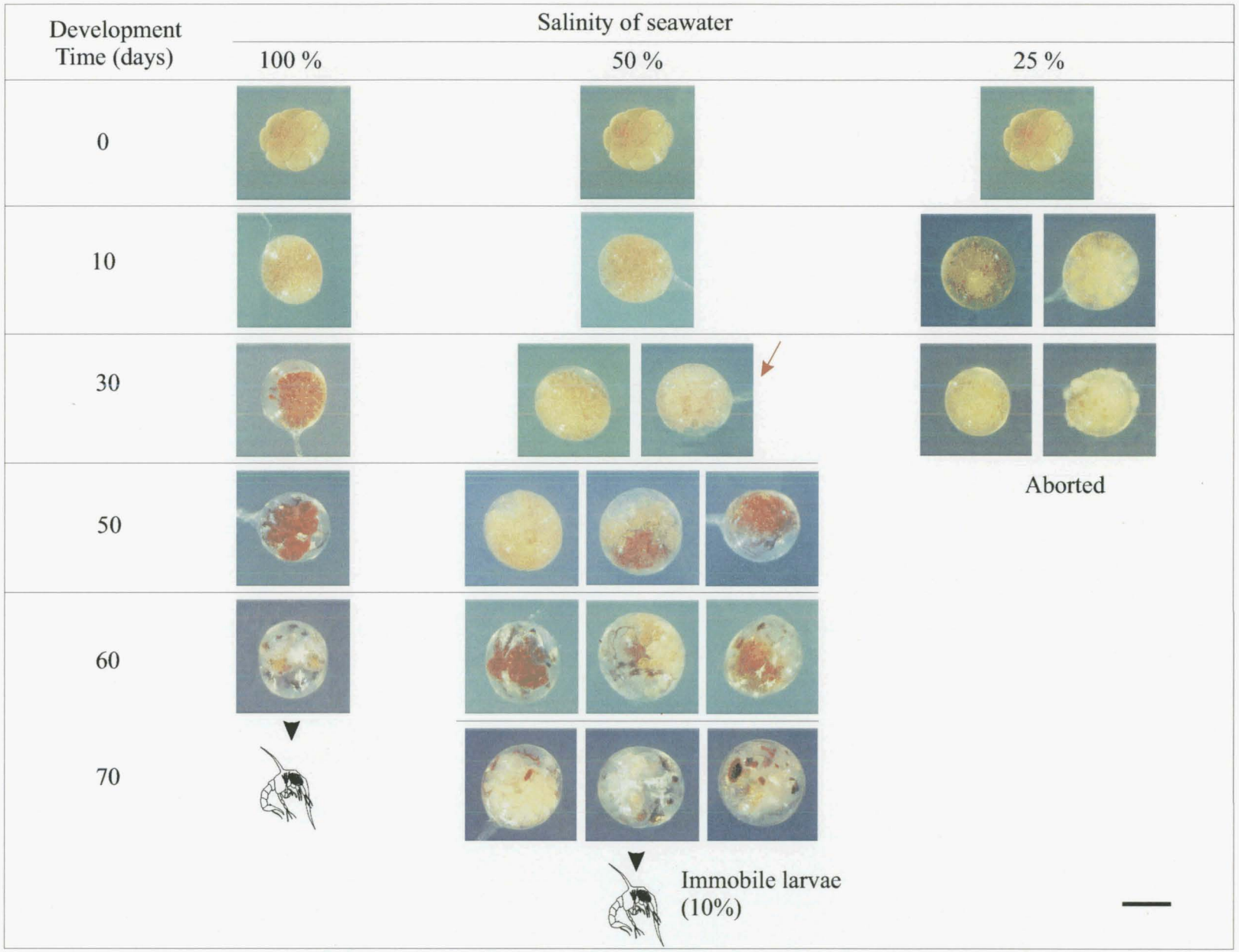



**Figure 3.7** Development and hatching success of embryos of *H. edwardsii* in three different salinities from stage 1 (Cleavage - Blastula) of development in the laboratory tidal tank system at 15 °C. Red arrow indicates when delayed development began. Scale bar = 300 µm.

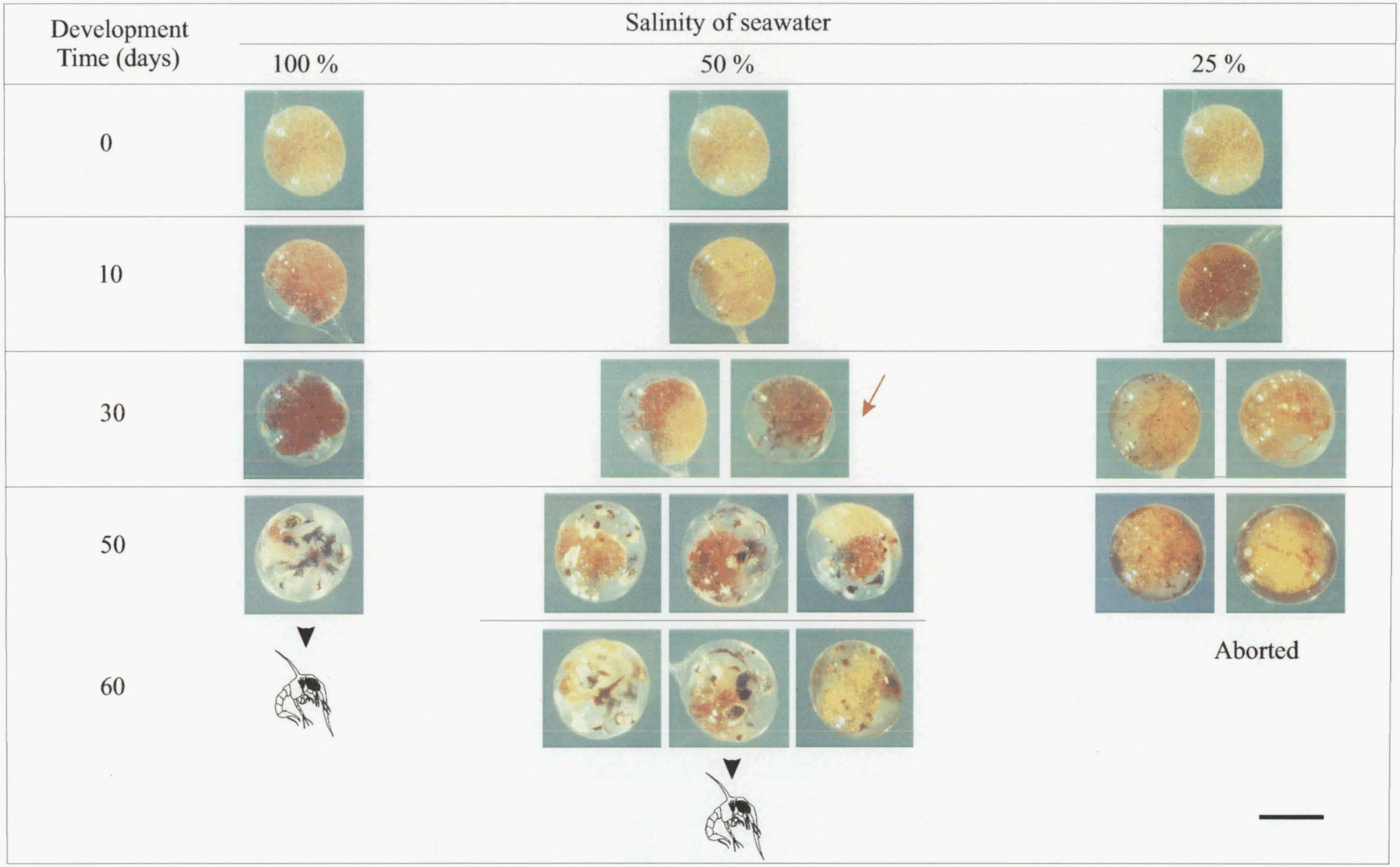



**Figure 3.8** Development and hatching success of embryos of *H.edwardsii* in three different salinities from stage 2 (Gastrula) of development in the laboratory tidal tank system at 15 °C. Red arrow indicates when delayed development began. Scale bar = 300μm.

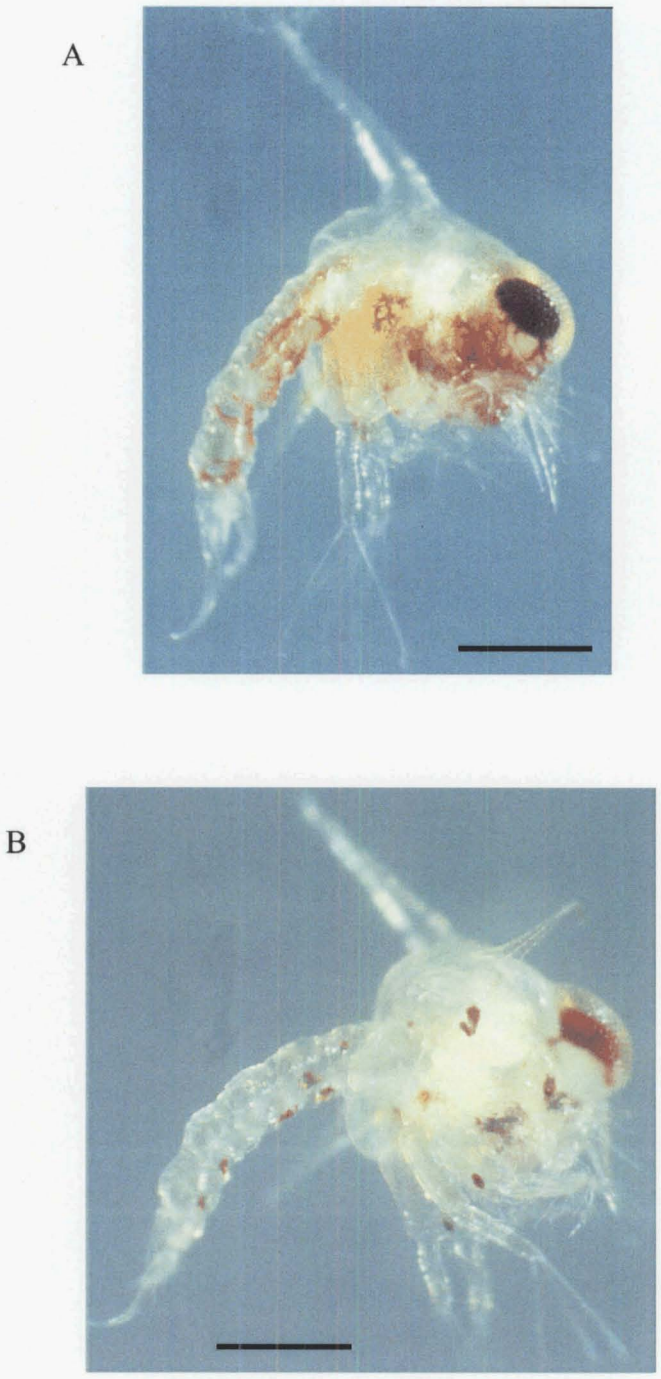



**Figure 3.9** Hatched larvae of *H. edwardsii* developed in (A) 100% seawater and (B) 50% seawater, showing the increased depletion of yolk in dilute seawater. Scale bars = 300 μm.

**(b) Changes in the total volume of embryos**

Total volume of embryos incubated *in situ* increased during development. Figure 3.10 shows the changes in the total volume of a single egg (nL) developed in 100% seawater and dilute seawater from stage 1 and stage 2. The volume changes in dilute seawater were small except in the case of the embryos commenced at stage 1 in 25% seawater and they were subsequently aborted.

**(c) Osmoregulation of embryos**

Embryos of *H. edwardsii* were hyperosmotic to the medium throughout the development time. For embryos introduced at stage 1, total egg osmolality decreased rapidly during the first few days at 50% and 25% salinities, and then plateaued (Figure 3.11). For stage 2 embryos, the rate of fall of osmolalites in dilute seawater was slower and it gradually plateaued but at a similar value to the stage 1 embryos.

**Development of embryos of *H. crenulatus* continuously exposed to low salinities**

As for *H. edwardsii*, the effect of continuous exposure to dilute seawater on the development of embryos was observed by introducing ovigerous crabs carrying embryos either at stage 1 or stage 2 to 100%, 50% and 25% seawater until hatching.

**(a) Viability and hatching success**

Stage 1 embryos developed in 100% seawater successfully hatched after 37 days (Figure 3.12). As for *H. edwardsii,* in 50% seawater, rates of development of stage 1 embryos were generally similar but development was delayed and arrested at stage 5. Delayed hatching of embryos as immobile larvae was observed in 50% seawater after 43 days. In 25% seawater development did not proceed beyond stage 2 and they did not hatch and the majority of female crabs gradually aborted their embryos after 18 days.

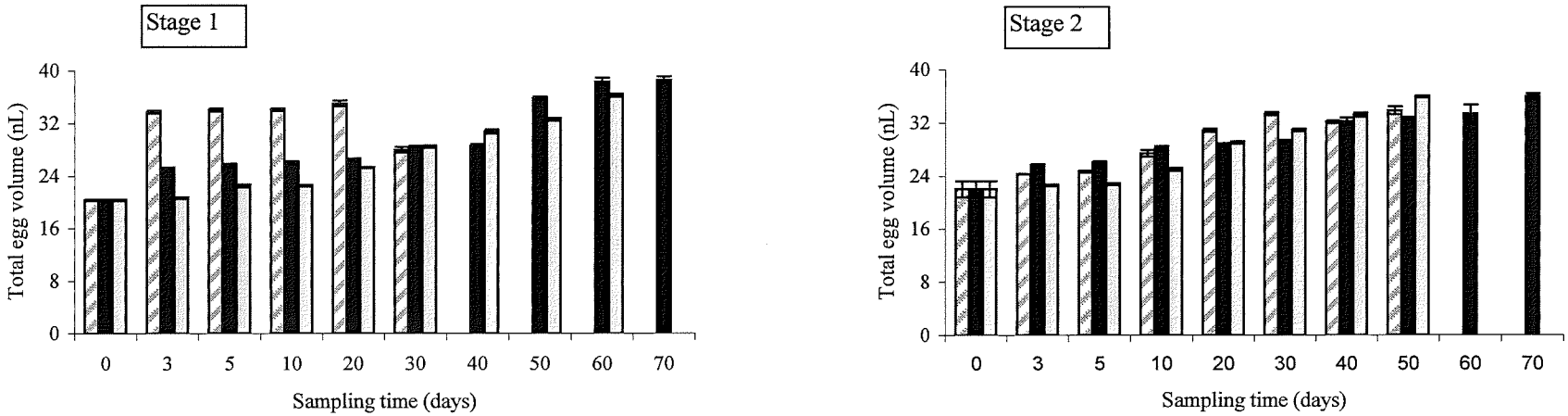



**Figure 3.10** Total egg volume (nL) of *Hemigrapsus edwardsii* introduced to experimental salinities either at stage 1 or at stage 2 at 15 °C. (▨ 25% seawater, ■ 50% seawater and □ 100% seawater). Values are mean ± S.E.M.. (N = 5 for each point).

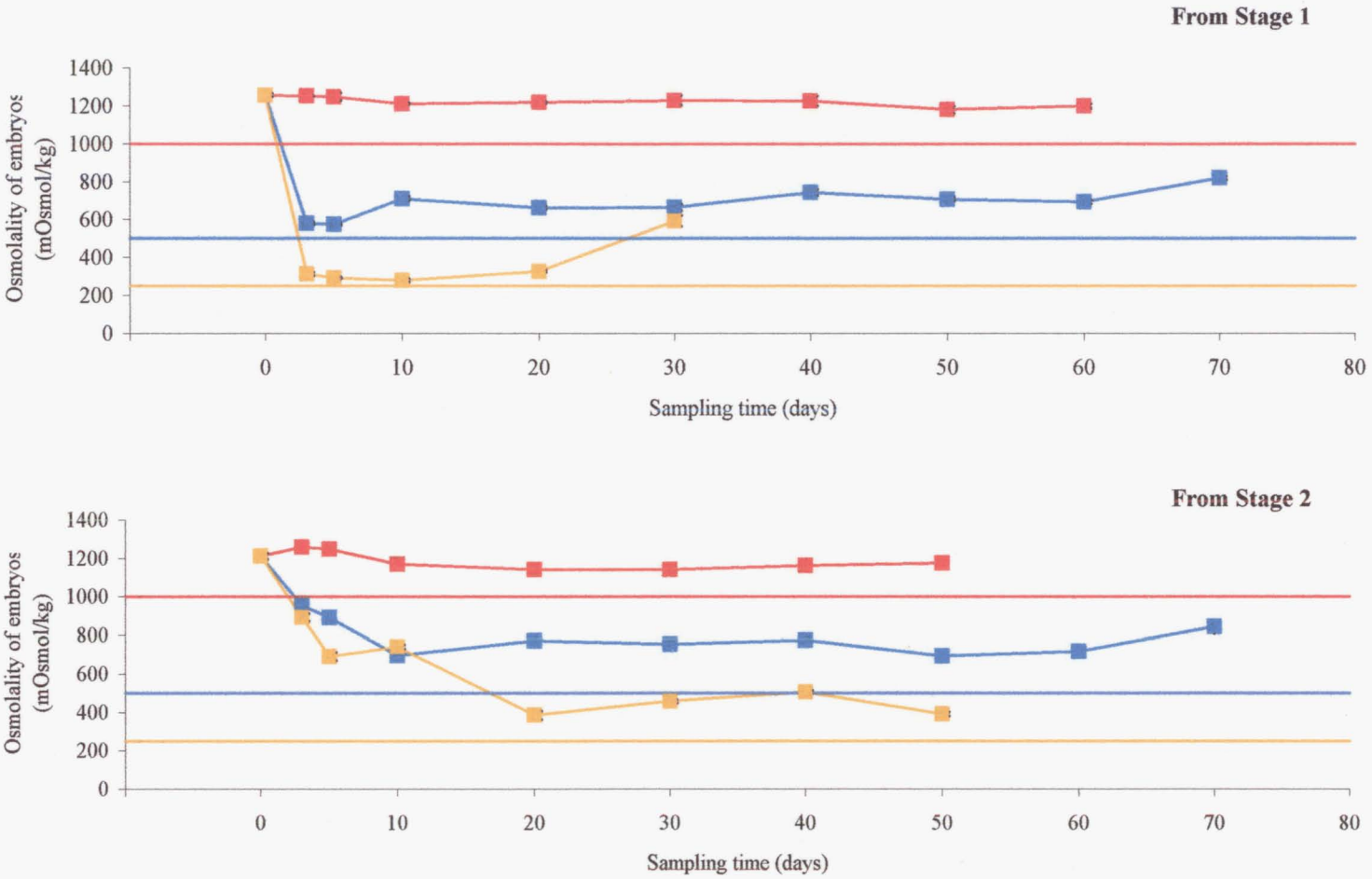



**Figure 3.11** Osmolalities of embryos incubated by ovigerous *Hemigrapsus edwardsii* and transferred from 100% seawater to dilute seawater either at stage 1 (0 - 5 days of development time) or at stage 2 (5 - 10 days) at 15 °C. ( 100% seawater, 50% seawater and 25% seawater). Horizontal lines indicate the osmolalities of the three experimental media. Values are mean ± S.E.M.. ( N= 5 for each point).

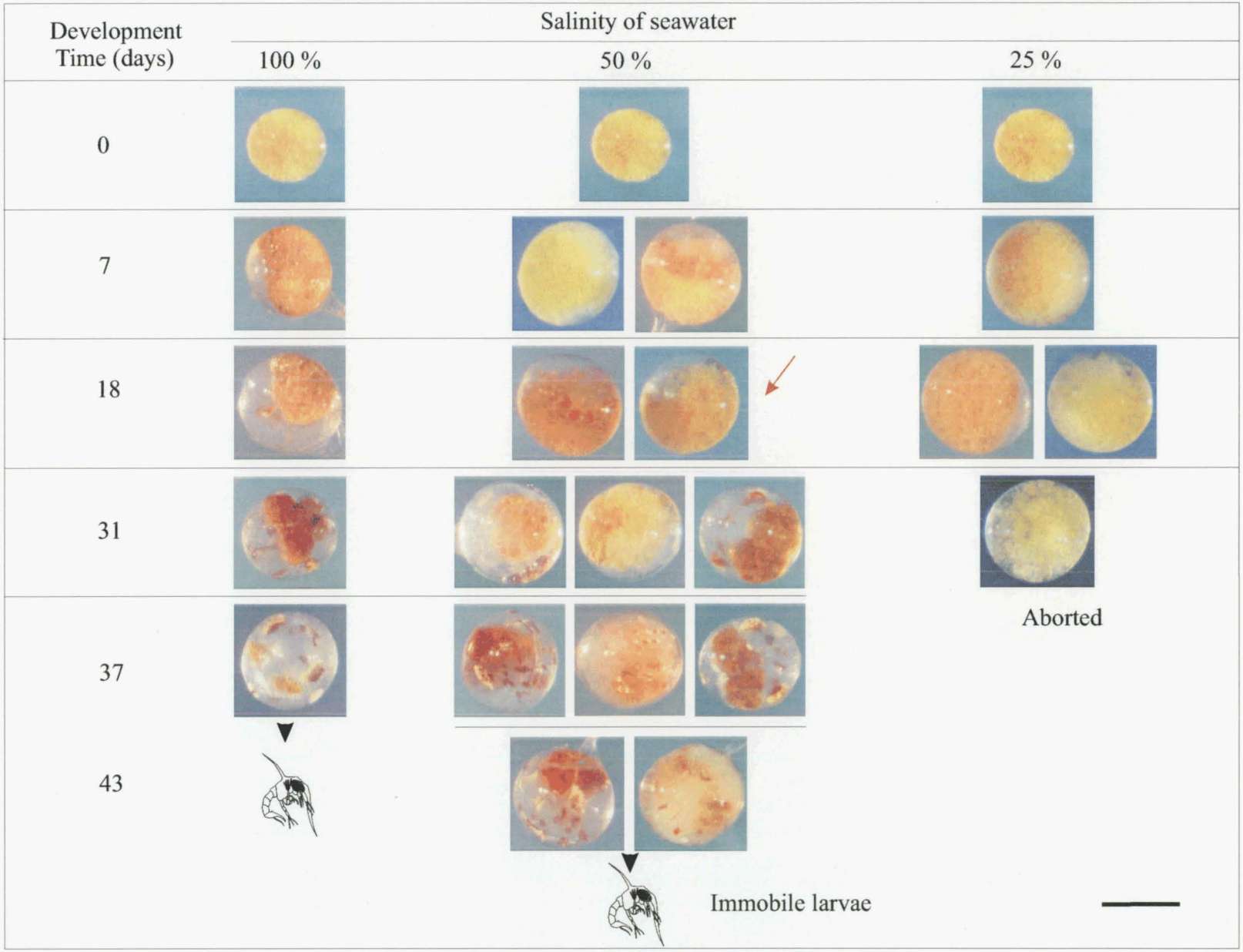



**Figure 3.12** Development and hatching success of embryos of *H. crenulatus* in three different salinities from stage 1 (Cleavage - Blastula) of development in the laboratory tidal tank system at 15 °C. Red arrow indicates when delayed development began. Scale bar = 300 µm.

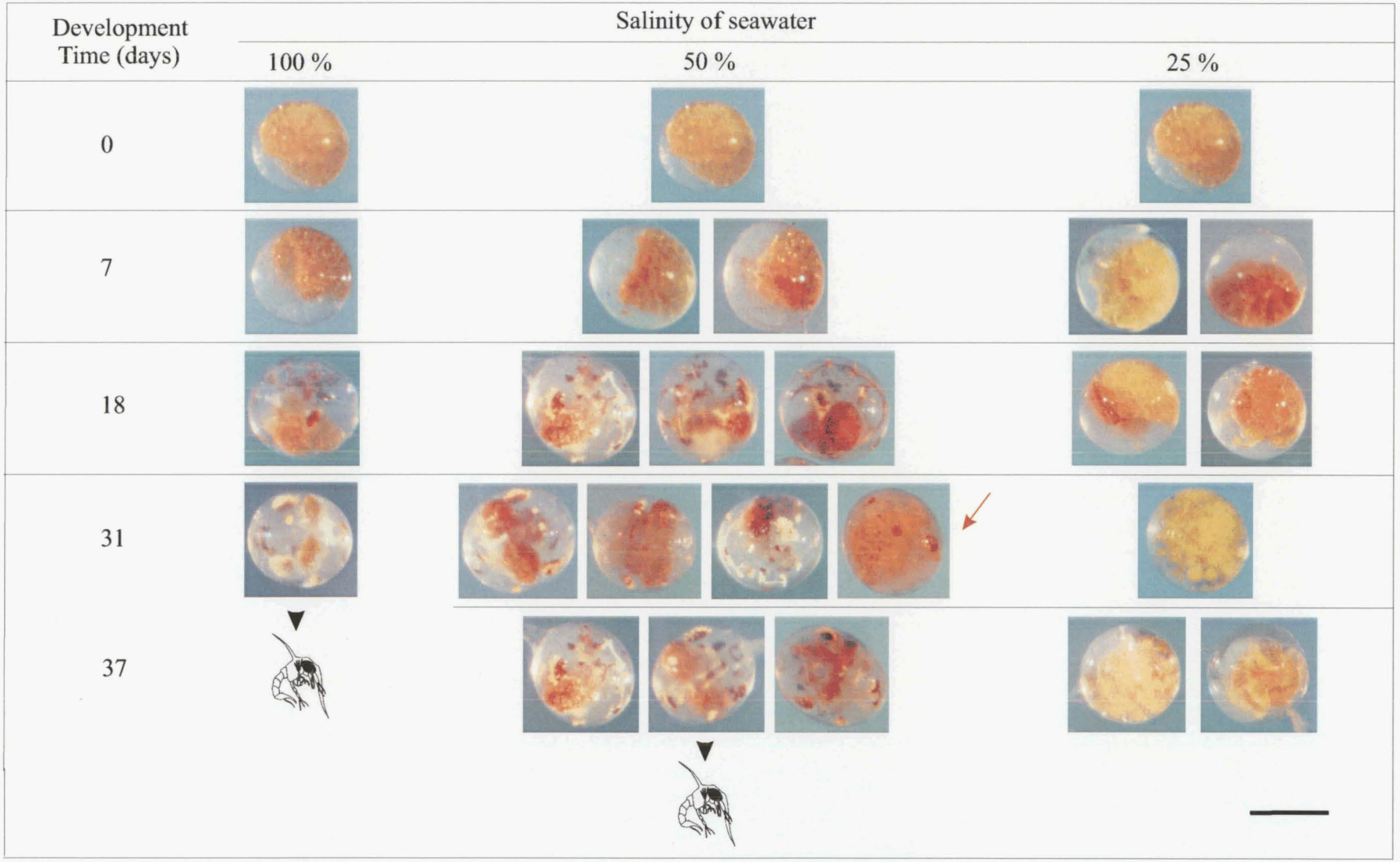



**Figure 3.13** Development and hatching success of embryos of *H. crenulatus* in three different salinities from stage 2 (Gastrula) of development in the laboratory tidal tank system at 15 °C. Red arrow indicates when delayed development began. Scale bar = 300 µm.

Figure 3.13 shows the morphological changes and the development of embryos from stage 2 in 100%, 50% and 25% seawater. Embryos successfully hatched after 31 days in 100% seawater. It was found that in 50% seawater the development of stage 2 embryos were similar to 100% seawater but delayed and hatched successfully as active larvae after about 37 days. As for *H. edwardsii*, changes in the colour of the yolk cells of the embryos developed in 50% seawater was observed, and no critical measurements were done on the rate of depletion of yolk in these embryos. As in stage 1 embryos, stage 2 embryos did not hatch successfully in 25% seawater, even though the development proceeded further and abortion of embryos was recorded after about 37 days.

**(b) Changes in the total volume of embryos**

Figure 3.14 shows the changes in the total volume of embryos developed from stage 1 and stage 2 in 100%, 50% and 25% seawater. A similar pattern was seen for *H. crenulatus* embryos with that of *H. edwardsii*. Total egg volume increased during development. There was an increase in the egg volume of stage 1 embryos in dilute seawater as for *H. edwardsii* and they did not develop and aborted after about 18 days. The volume of eggs introduced at stage 2 was not affected in dilute seawater.

**(c) Osmoregulation of embryos**

Embryos of *H. crenulatus* were also hyperosmotic to the external medium throughout their development (Figure 3.15). As for *H. edwardsii*, osmolality of embryos dropped after few days of introduction to low salinities. The rates of fall of osmolalities were more sharp for stage 1 embryos compared to stage 2 in 50% and 25% seawater and it gradually plateaued with time and maintained hyperosmotic state throughout development.

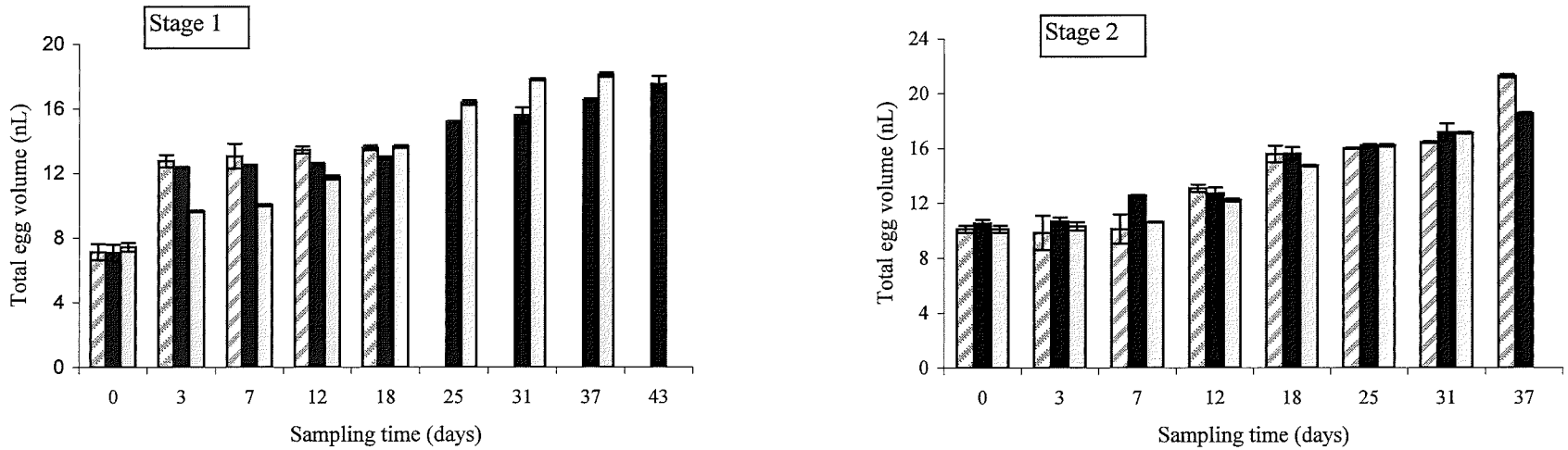



**Figure 3.14** Total egg volume (nL) of *Hemigrapsus crenulatus* introduced to experimental salinities either at stage 1 or at stage 2 at 15 °C. ( ▨ 25% seawater, ■ 50% seawater and □ 100% seawater). Values are mean ± S.E.M.. (N = 5 for each point).

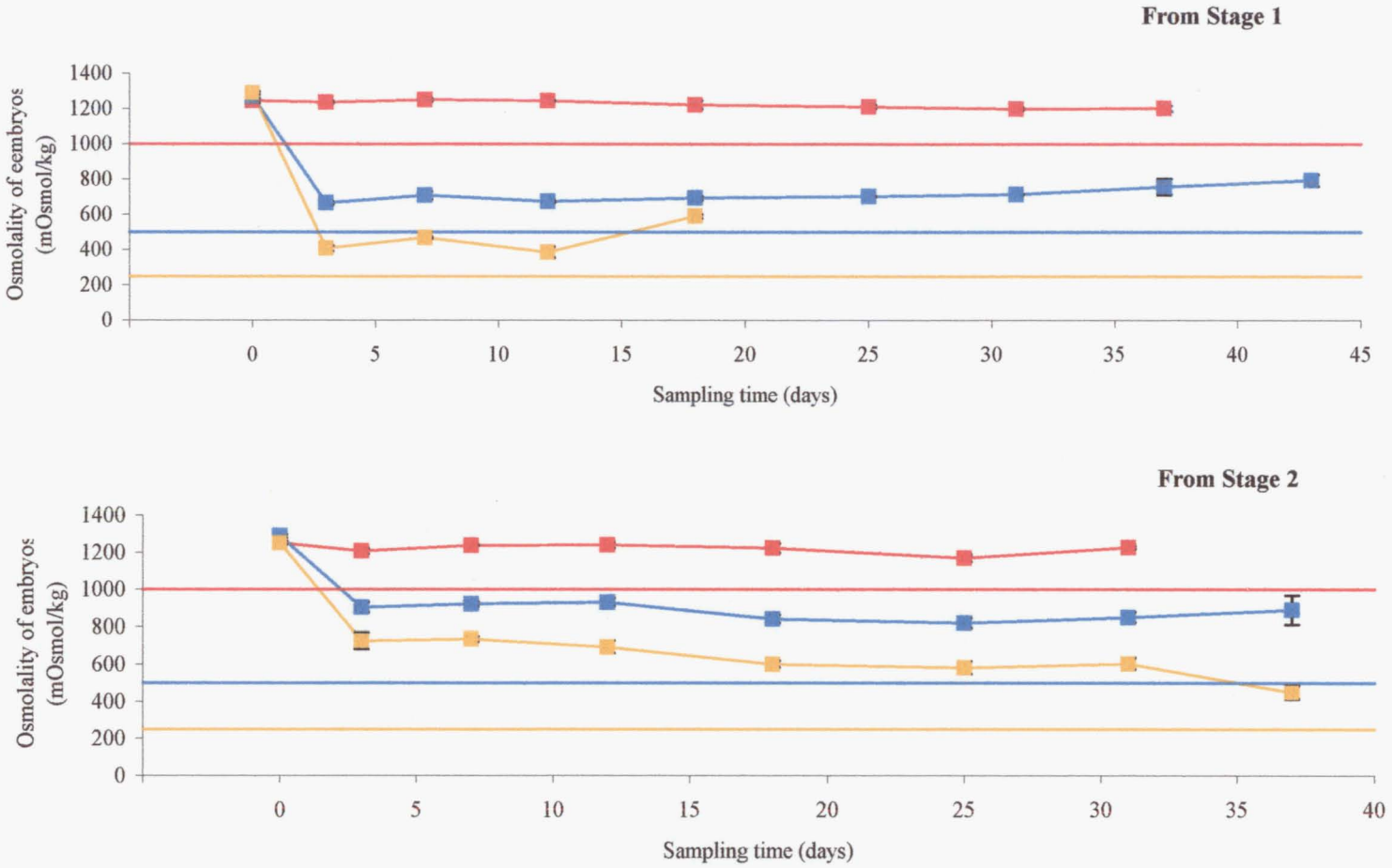



**Figure 3.15** Osmolalities of embryos incubated by ovigerous *Hemigrapsus crenulatus* and transferred from 100% seawater to dilute seawater either at stage 1 (0 - 2 days of development time) or at stage 2 (3 - 8 days) at 15 °C. ( 100% seawater, 50% seawater and 25% seawater). Horizontal lines indicate the osmolalities of the three experimental media. Values are mean ± S.E.M.. ( N= 5 for each point).

## DISCUSSION

The present investigation confirms that the early life history stages of both *Hemigrapsus edwardsii* and *H. crenulatus* are capable of survival and development in a fluctuating salinity environment. The salinity tolerance varied with the developmental stage for both species. Except for embryos at cleavage/blastula stages (stage 1), all stages could survive in dilute seawater for many hours when exposed acutely (Figures 3.1 & 3.2). This type of relationship was observed in the developing embryos of *Heterozius rotundifrons* and *Cyclograpsus lavauxi*, i.e. late stage embryos were more tolerant of reduced salinity than early stage embryos (Leelapiyanart, 1996).

In the long term salinity tolerance experiment, pre-gastrula stage (stage 1) embryos of both *H. edwardisii* and *H. crenulatus*, were unable to develop and hatch normally in 50% sea water and development did not proceed beyond stage 2 in 25% sea water. However, when ovigerous crabs of either species with stage 2 embryos were exposed to 50% seawater, development and hatching were successful although slightly delayed. These observations are comparable to those found by Clark (1987) for *H. crenulatus* who reported successful brood development between 18-36 ppt salinity with development time increasing with decreasing salinity.

In a similar study of Bas and Spivak (2000) with the embryos of two southwestern Atlantic estuarine grapsid crabs *Chasmagnathus granulata* and *Cyrtograpsus angulatus*, normal embryonic development occured at salinities between 12 ppt and 40 ppt. Outside these extremes, development was arrested. Bas and Spivak (2000) also noted that embryos of both species acquired tolerance to extreme salinities (3 and 44 ppt) after two or three days following egg extrusion.

When effects of salinity change on the subsequent developmental stages of *H. edwardsii* and *H. crenulatus* are compared, ontogenetic changes in salinity tolerance are discernible. A plausible explanation for high survival rates at late stages of embryonic development may be the capability to osmoregulate. This is consistent

with the observation of putative osmoregulatory structures in embryos at the later developmental stages (se Chapter 2) and as discussed by Bouricha *et al.* (1994).

This study has demonstrated that the embryos at all stages, beyond stage 1, of both *H. edwardsii* and *H. crenulatus* were hyperosmotic to the external medium in all low salinities tested (Figures 3.5 & 3.6). The osmolalities of crab embryos decreased with the decrease of salinity of seawater, and the solvent volume increased as water moved into the egg cell by osmosis. It appears that the capacity to osmoregulate commences at the gastrula stage for these embryos. Gastrulation marks the critical stage for these embryos where transition from osmoconforming to hyper-osmotic regulation occurs. Similarly, Felder *et al.* (1986) and Bouricha *et al.* (1994) suggested that the capacity to osmoregulate can occur at any stage of development. However, these two *Hemigrapsus* species develop a capacity for osmoregulation much earlier than was proposed in those studies.

In the long term exposure study, it was found that embryos were hyperosmotic to the medium throughout the development time (Figures 3.11 & 3.15). Total egg osmolality decreased rapidly during the first few days of introduction of cleavage stages to the low salinities and then plateaued whereas the rate of fall of osmolality for post-gastrula stage embryos in low salinities was slower. These observations suggest that the osmotic shock which these early stage embryos faced during these important morphogenetic stages (cleavage, gastrulation) may be the reason for the failure of the cleavage stages to develop normally.

During ontogeny of several crustacean species, a strong correlation has been observed, between an increasing ability to osmoregulate and an improving salinity tolerance (Charmantier & Charmantier-Daures, 1991, 1994; Charmantier *et al.*, 1998; Leelapiyanart, 1996; Morritt & Spicer, 1995). Charmantier *et al.* (2001) in a review study of *Homarus* sp, documented that when they were exposed to low salinity, the capacity to osmoregulate changes during development. These embryos were osmoconformers and it was inferred that they were osmotically protected by the egg membranes. Larvae of these species were also osmoconformers, but the pattern of osmoregulation changes at metamorphosis to hyper-regulation, which was then retained throughout the later stages up to the adult stage.

Therefore, among the few species studied to date, an ontogenic increase both in osmoregulation and salinity tolerance appears to be common. The physiological capabilities of embryos of *Hemigrapsus* species at different stages of development indicate that survival through the cleavage stages requires the avoidance of diluted seawater by early ovigerous crabs. In contrast, post-gastrula embryos are more tolerant of salinity change and both species of adult crabs are excellent osmoregulators and are able to survive prolonged dilution (Jackson 1976; Bedford & Leader, 1978, 1979; Leader & Bedford, 1978). It would thus be of interest in future studies to determine whether there are observable differences in the behavioural responses between ovigerous and non-ovigerous crabs, or between those crabs carrying embryos at different developmental stages when they encounter dilute seawater on the shore.

In conclusion, it can be said that the developing embryos of *H. edwardsii* and *H. crenulatus* themselves have the ability to survive, to maintain a hyperosmotic condition, and to resist swelling in dilute seawater for a long period. The current hypothesis that decapod embryos are osmoconformers is no longer applicable to these two crab species. An ontogenic increase both in osmoregulation and salinity tolerance was documented, and normal development and successful hatching in dilute sea water were observed from post gastrula stage of both species. In these intertidal crabs, the embryos develop externally where they are subjected to variations in salinity. Clearly, to survive in such situations, the early acquisition of osmoregulatory capacity is an advantage. However, these embryos do not yet posses the organs responsible for excretion and water and ion uptake in adults (antennal organ, gills). Possible mechanisms of osmoregulation employed by these embryos and the permeability of egg membranes are studied and discussed further in Chapters 5 and 6.

# CHAPTER FOUR

# ION REGULATION BY CRAB EMBRYOS

*Summary*

- The overall concentrations of the four cations $Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$ increased with embryonic development and decreased as the salinities decreased.
- Sodium and potassium were found to be the main cations in these embryos.
- All four cations were major osmotic effectors in the embryos of *H. crenulatus* but contributed only about half of the total osmolality.
- The average concentrations of all four cations were better regulated than was total osmolality. This indicates the involvement of other osmolytes in osmoregulation.

## INTRODUCTION

Ion regulation may be defined as the capacity of an organism to build up and maintain specific ionic gradients between cell fluids, body cavity fluids and external medium. In this definition, the term 'cell fluids' refers to intra- and intercellular liquids, and the term 'body cavity fluids' to blood, lymph or other liquids of body cavities (Kinne, 1971).

The factors which create and maintain ionic concentration differences between an animal and its environment have been the subject of many investigations. Potts & Parry (1963) reported that the occurrence and extent of the concentration differences are the result of the interaction of both passive and active factors. The passive factors are; 1. the permeability of the animal's body wall to water and solutes, 2. the presence of protein in the body fluids which may produce Donnan effects, and which may bind some ions in indiffusible complexes. The active factors are; 3. the excretion of salts and water from the body, 4. the active uptake of salts, and possibly water, in the gut and at the body surface. From the Gibbs-Donnan theory, it follows that animal cells which have easily distensible plasma membranes should necessarily undergo swelling and lysis unless some active mechanisms are present to maintain the

activity of water in the intracellular medium close to that of the extracellular fluid (Gilles, 1979).

Prosser (1973) has noted that there are two situations in which active ion transport is required; regulation of intracellular ionic concentrations when the extracellular fluids have a very different ionic composition, and regulation of stable body fluid concentrations when the external environment has a different pattern. All cells have some capacity for cell volume regulation when confronted with a hypoosmotic stress. It is known that when there is a change in the concentration of the medium, an animal may behave as either an osmoconformer or an osmoregulator (Refer Chapter 3). The basis of this physiological response is an extrusion of intracellular osmotic solutes. In the tissues and cell types studied up to now, the major osmotic effectors implicated in volume regulation are of two types; the inorganic monovalent ions $Na^+$, $K^+$ and $Cl^-$; divalent ions $Mg^{2+}$, $Ca^{2+}$ and $SO_4^{2-}$ on the one hand and different organic solutes of low molecular weight (amino acids) on the other hand. The relative contribution of each solute type varies from species to species and, perhaps, from cell type to cell type (Gilles, 1979; Pierce, 1982). Therefore, it is generally accepted that organisms employ a combination of inorganic ion transport and organic osmolyte deployment to match intracellular and extracellular osmolalities, thus reducing the energy required to maintain cell volume (Wolcott, 1991).

Charmantier *et al.*, (1998) in a review have documented that the capacity to osmoregulate is based on efficient ionic regulation. The role of inorganic ions in osmotic and volume regulation has been studied on a variety of crustacean tissues and cell types (Dehnel & Carefoot, 1965, 1967; Engelhardt & Dehnel, 1973; Gifford, 1962; Kevers *et al.*, 1979; Piller *et al.*, 1995; Siebers *et al.*, 1985; Terwilliger & Brown, 1993; Zanders, 1980; Flik & Haond, 2000; Wheatly & Gannon, 1995; Zanders & Rodriguez, 1992). In the axons of *Carcinus*, the regulation of intracellular $K^+$ plays an important role in limiting the swelling (Kevers *et al.*, 1979).

Ion regulation as an integral part of the osmotic and volume regulation of embryos of different species has been investigated by Hayes *et al.*, 1946; Kalman, 1959; Potts & Rudy, 1969; Shen & Leatherland, (1978); Shephard, 1987; Shephard & Mc Williams,

1989 (freshwater fish) ; Brown & Terwilliger, 1992; Leelapiyanaart, 1996 ( crabs); Beadle, 1969 ; Taylor, 1977 (mollusc).

It has been shown in most of those studies that during acclimation to different salinities, the cells successfully cope with osmotic stresses, the amplitude of which is dependent on the regulatory ability of the osmotic effectors. The presence of an ontogenic change in both ionic and osmotic regulation in the larval stages of *Cancer magister* has been demonstrated by Brown and Terwilliger (1992).

It is shown in Chapter 3, that the developing embryos of *H. crenulatus* can maintain and regulate their internal osmotic concentration when exposed to dilute seawater. It was also noted that except for the stage 1 embryos, developing embryos have the capacity to regulate volume in hyposaline waters. As noted above, previous studies have demonstrated that the occurrence of osmoregulation is based on efficient ion regulation, and, it is of particular interest to consider the ability of these developing embryos to regulate ions under salinity stress conditions in relation to osmoregulation. Therefore, in this study, embryos of *H. crenulatus* at different developmental stages were acclimated for 24 h to a range of salinities and the total contents and concentrations of cations ($Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$) were determined. It is shown that these inorganic ions act as important osmotic effectors and show individual patterns of regulation that differ at different developmental stages.

## MATERIALS AND METHODS

### Maintenance of ovigerous crabs

Ovigerous crabs of *Hemigrapsus crenulatus* carrying embryos at different developmental stages were collected from the intertidal zones of Avon-Heathcote Estuary during the spawning period from August to January were maintained in the aquarium, Department of Zoology at 15 °C.

### Experimental design

Concentration of cations sodium, potassium, calcium and magnesium were measured using the embryos of *H. crenulatus* at different stages of development. Changes in the total cation concentration in dilution were investigated by exposing detached embryos at different developmental stages to a range of salinity (1% to 100% seawater) for 24 h.

### Experimental media

Seawater at different salinities (100%, 50%, 10% and 1%) was prepared by diluting "Instant Ocean" (Aquarium Systems, Inc.) salt with tap water. Concentrations of the cations ($Na^+$, $K^+$, $Mg^{2+}$ and $Ca^{2+}$) in the experimental media are shown in Table 4.1 (calculated using tables of Barnes, 1954).

**Table 4.1** Cation concentration in the experimental media

| Salinity (% seawater) | Osmolality ($mOsmol.L^{-1}$) | Ion Concentration ($mmol.L^{-1}$) | | | |
|---|---|---|---|---|---|
| | | $Na^+$ | $K^+$ | $Mg^{2+}$ | $Ca^{2+}$ |
| 100 | 1050 | 489.4 | 10.4 | 55.8 | 10.7 |
| 50 | 525 | 244.7 | 5.18 | 27.9 | 5.32 |
| 10 | 105 | 48.9 | 1.04 | 5.57 | 1.06 |
| 1 | 10.5 | 4.89 | 0.104 | 0.56 | 0.11 |

**Measurement of cations in developing embryos**

Embryos at different developmental stages defined in Chapter 2 were detached from the ovigerous crabs reared in the aquarium at different times and exposed to above experimental media (100%, 50%, 10% and 1% seawater) for 24 h. After 24 hours of exposure time, embryos were blotted dry with filter papers, weighed (about 0.1 – 0.2 g) and transferred to 16mm diameter capped polyethylene tubes. Then the embryos were digested with 0.5 ml of conc. $HNO_3$ for 24 h in a water bath at 50 °C. (Preliminary experiments were carried out comparing metal ion recovery using dry ashing and wet digestion of eggs and, on the basis of those trials, the latter method was chosen). Digested embryos were dissolved in 25 ml of double distilled water and samples of 0.5 ml for $Na^+$ and $K^+$ and 5.00 ml for $Mg^{2+}$ and $Ca^{2+}$ were taken, and cation concentrations were measured by using a Varian Techtron 1200 Atomic Absorption Spectrophotometer using an air-acetylene flame for atomisation.

Samples were diluted to 10 mL using suitable ionisation suppressants (9.5 mL of 1.334 g/L of $CsCl_2$ for $Na^+$ and $K^+$) and releasing agents (5.00 mL of 5g/L of $LaCl_3$ for $Mg^{2+}$ and $Ca^{2+}$). All cations were measured in the absorption mode at wavelength 589, 766.5, 422.7 and 285.2 nm for $Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$ respectively. Calibration standards containing the same additives were on the following ranges for $Na^+$(19 – 95 $\mu mol.L^{-1}$); $K^+$ (10 – 50 $\mu mol.L^{-1}$); $Ca^{2+}$ (8 - 40 $\mu mol.L^{-1}$); & $Mg^{2+}$ (20- 100 $\mu mol.L^{-1}$).

Care was taken to avoid contamination from the skin and dust in the air. The highest purity chemicals and double distilled water were used throughout and all glassware was acid soaked and rinsed in distilled water.

Subsamples of embryos were counted, weighed and the diameters measured to estimate single egg mass and volume (Chapter 2). Cation concentrations are expressed as mmoles per litre ($mmol.L^{-1}$). The estimated total Na contents (nmol) are given in Chapter 6 (Table 6.3).

**Calculation of contribution of inorganic ions to the osmolality of embryos of *H. crenulatus***

The component of osmolality due to measured cations and associated anions in the embryos of *H. crenulatus* was calculated using the data from Table 3.6 to 3.10 and 4.3 to 4.6 for each developmental stage and salinity.

The contribution of cations was calculated as the sum of the molarities of the measured ions ($Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$). It was assumed that all the cations were balanced by monovalent anions. Thus the molarity each cation was multiplied by its valency and these values added to the cations. This sum was then multiplied by the osmotic coefficient of 0.9 (Potts and Parry, 1963; Prosser, 1973). Finally, assuming that ions were distributed only in the solvent volume in these eggs, the osmotic contribution of ions was adjusted using the total egg volume and solvent volume for each stage and salinity.

**Statistical Analysis**

Concentrations of cations in the embryos are expressed as mean ± S.E.M. ($mmol.L^{-1}$). Differences in cation concentrations among developmental stages in 100% seawater (control) were tested by one-way ANOVA (stage was used as the categorical predictor factor). The effect of dilution on the cation concentrations in each development stage was also tested by one-way ANOVA (salinity as the categorical predictor factor). Subsequent multiple comparisons of means were performed using Tukey HSD post-hoc test. Differences are reported as statistically significant when $P < 0.05$. The programme STATISTICA 6 was used for the statistical analysis.

# RESULTS

## Ionic composition of embryos of *H. crenulatus*

Table 4.2 presents the mean concentration of four cations in embryos, expressed in terms of total egg volume, for the 5 developmental stages in 100% seawater. Except in the case of sodium, there were significant differences in ion concentrations among the stages (one way ANOVA, see results table below and Tukey post-hoc comparisons of means in Table 4.2). The potassium concentration of embryos at stage 1 was about four times higher than that in the medium, and increased significantly to about ten times by stage 2. Magnesium ion concentration decreased during development of embryos. Values (± SE) indicate that there was about two-fold decrease in the magnesium concentration from stage 1 to stage 5 embryos. Sodium and Potassium were the main cations in the embryos (Figure 4.1 & 4.2).

Results of one way ANOVA, testing the effect of the factor stage of development on the cation concentration on *H. crenulatus* embryos in 100% seawater are shown in the table below.

| Cation | F statistics | *P* |
|---|---|---|
| Sodium | $F_{(1), 4, 26} = 0.72$ | 0.587295 |
| Potassium | $F_{(1), 4, 25} = 9.47$ | 0.000084 |
| Calcium | $F_{(1), 4, 25} = 3.22$ | 0.029087 |
| Magnesium | $F_{(1), 4, 25} = 16.344$ | 0.000001 |

See Table 4.2 for Tukey post-hoc comparison of means.

## Ionic regulation of embryos of *H. crenulatus*

The overall concentrations of all four cations decreased as the salinities decreased, in all stages (Figures 4.3 & 4.4).

### $Na^+$ concentration

Table 4.3 presents the mean values (±SEM) for sodium concentration in embryos of different stages after 24 h in different salinities. It is shown that sodium concentration

**Table 4.2** Concentrations of cations sodium, potassium, calcium & magnesium in different embryonic stages of *Hemigrapsus crenulatus* in 100% seawater at 15 °C. Values are mean ± SEM of 5 or more replicates. Means within each column with different letter labels (a,b,c,d & e) are statistically different at $P < 0.05$.

| | Cation concentration ($mmol.L^{-1}$) | | | |
|---|---|---|---|---|
| | Sodium | Potassium | Calcium | Magnesium |
| Medium (100% seawater) | 489.4 | 10.4 | 10.7 | 55.8 |
| Stage of development | | | | |
| Stage 1 | 112.52 ± 6.35 | 47.07 ± 8.56 [a] | 6.36 ± 0.58 [a] | 53.28 ± 5.3 [a] |
| Stage 2 | 104.06 ± 13.5 | 106.8 ± 10.32 [b] | 7.54 ± 0.53 [a] | 40.62 ± 1.96 [b] |
| Stage 3 | 117.52 ± 8.3 | 124.72 ± 13.64 [c] | 8.97 ± 0.61 [b] | 34.71 ± 2.42 [c] |
| Stage 4 | 125.03 ± 6.19 | 113.83 ± 4.33 [d] | 8.64 ± 0.40 [a] | 29.33 ± 1.01 [d] |
| Stage 5 | 119.31 ± 5.48 | 98.03 ± 4.75 [e] | 7.73 ± 0.17 [a] | 25.55 ± 1.05 [e] |

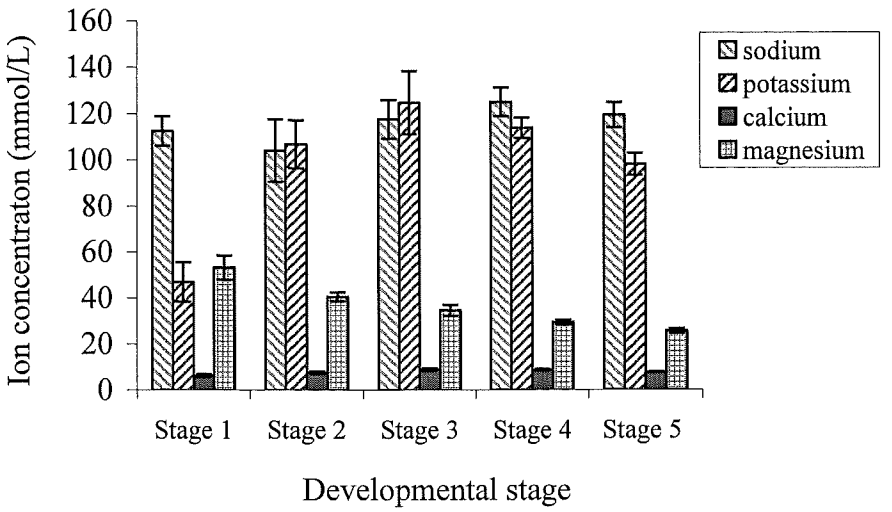



**Figure 4.1** Changes in the cation concentration in embryos of *H. crenulatus* at different stages of development in 100% seawater at 15 °C. Values are mean ± SEM. Statistical differences at $P < 0.05$ are indicated in the table.

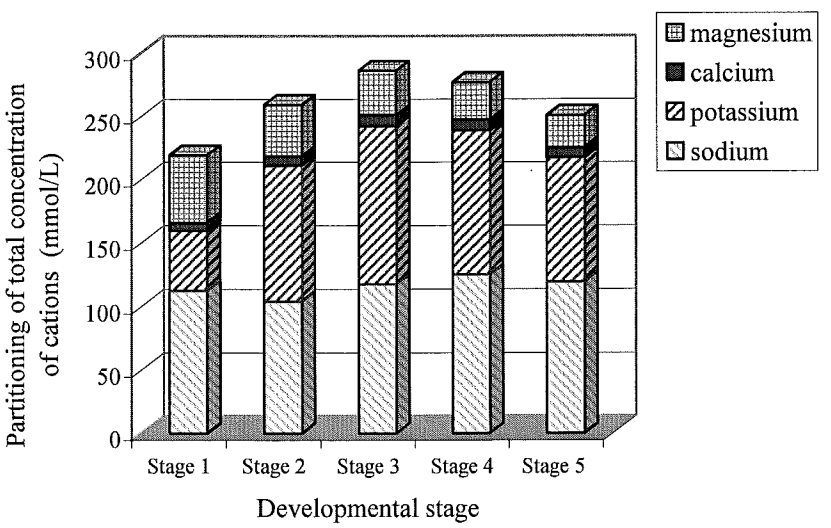



**Figure 4.2** Partitioning of total concentration of cations in embryos of *H. crenulatus* at different developmental stages in 100% seawater at 15 °C.

increased with the development but significantly decreased with the decreasing salinity for all five stages (ANOVA, Tukey, post hoc). $Na^+$ concentration in embryos of all five developmental stages was hypo-ionic in 100% and 50% seawater. Except for pregastrula stage embryos (stage 1), a strong hyper-regulation of sodium in 10% and 1% seawater was recorded for post gastrula embryos.

Results of one way ANOVA, testing the effect of the factor salinity on sodium ion concentration on *H. crenulatus* embryos at each of 5 stages are shown in the table below.

| Stage | F statistics | *P* |
|---|---|---|
| 1 | $F_{(1), 3, 19} = 188.3$ | 0.000000 |
| 2 | $F_{(1), 3, 25} = 22.2$ | 0.000000 |
| 3 | $F_{(1), 3, 30} = 47.9$ | 0.000000 |
| 4 | $F_{(1), 3, 28} = 52.9$ | 0.000000 |
| 5 | $F_{(1), 3, 16} = 3.05$ | 0.058749 |

See Table 4.3 for Tukey post-hoc comparison of means.

## $K^+$ concentration

Potassium was hyper-ionic for all developmental stages compared with ambient seawater (Table 4.4). There was a significant increase in the potassium ion concentration with the development of embryos from stage 2. Potassium concentration of embryos at stage 1 decreased significantly in dilute seawater (ANOVA, Tukey post hoc tests). Embryos at late developmental stages showed rather constant concentrations of potassium in all salinities and the effect of salinity on the potassium ion concentration was not detectable.

Results of one way ANOVA, testing the effect of the factor salinity on potasium ion concentration on *H. crenulatus* embryos at each of 5 stages are shown in the table below.

| Stage | F statistics | *P* |
|---|---|---|
| 1 | $F_{(1), 3, 21} = 87.9$ | 0.000000 |
| 2 | $F_{(1), 3, 29} = 1.5$ | 0.228930 |
| 3 | $F_{(1), 3, 32} = 0.4$ | 0.774021 |
| 4 | $F_{(1), 3, 27} = 0.8$ | 0.479524 |
| 5 | $F_{(1), 3, 16} = 0.6$ | 0.585168 |

See Table 4.4 for Tukey post-hoc comparison of means.

**Table 4.3** Concentration of sodium in different embryonic stages of *Hemigrapsus crenulatus* after 24 h in different salinities at 15 °C. Values are mean ± SEM of five or more replicates. Means with different letter labels (a,b,c &d) are statistically different @ $P < 0.05$.

| Salinity of seawater (%) | Sodium concentration ($mmol.L^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | Medium | Stage 1 | Stage 2 | Stage 3 | Stage 4 | Stage 5 |
| 100% | 489.4 | 106.46 ± 5.41 [a] | 129.82 ± 7.13 [a] | 118.07 ± 3.91 [a] | 142.94 ± 3.01 [a] | 131.26 ± 14.73 |
| 50% | 244.7 | 35.10 ± 3.56 [b] | 101.18 ± 2.73 [b] | 87.09 ± 3.12 [b] | 97.99 ± 4.66 [b] | 117.64 ± 8.61 |
| 10% | 48.9 | 6.06 ± 1.38 [c] | 85.13 ± 4.21 [c] | 78.27 ± 1.55 [c] | 88.35 ± 4.81 [c] | 96.97 ± 5.8 |
| 1% | 4.89 | 4.45 ± 0.99 [d] | 76.84 ± 0.84 [d] | 71.6 ± 2.41 [d] | 73.33 ± 3.63 [d] | 97.49 ± 6.25 |

**Table 4.4** Concentration of potassium in different embryonic stages of *Hemigrapsus crenulatus* after 24 h in different salinities at 15 °C. Values are mean ± SEM of five or more replicates. Means with different letter labels (a,b,c & d) are statistically different @ $P < 0.05$.

| Salinity of seawater (%) | Potassium concentration ($mmol.L^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | Medium | Stage 1 | Stage 2 | Stage 3 | Stage 4 | Stage 5 |
| 100% | 10.4 | 35.98 ± 2.15 [a] | 117.04 ± 2.70 | 93.00 ± 2.06 | 84.82 ± 2.04 | 83.69 ± 5.46 |
| 50% | 5.18 | 30.71 ± 1.96 [b] | 128.74 ± 6.81 | 95.82 ± 4.42 | 90.55 ± 3.70 | 89.18 ± 2.54 |
| 10% | 1.04 | 8.91 ± 1.09 [c] | 112.50 ± 8.68 | 99.44 ± 5.46 | 89.56 ± 4.19 | 82.07 ± 3.69 |
| 1% | 0.104 | 2.84 ± 0.97 [d] | 128.85 ± 6.32 | 95.75 ± 4.42 | 84.07 ± 4.09 | 84.40 ± 2.45 |

$Ca^{2+}$ concentration

Table 4.5 presents the calcium ion concentration in embryos at different stages after 24 h in different salinities. Calcium was hypo ionic to the medium throughout development in 100% seawater. There is a small increase in the calcium ion concentration with the development of embryos. In dilution, calcium was hyper-ionically regulated in all developmental stages, except for stage 1 embryos. A significant decrease (ANOVA, Tukey post hoc test) in the calcium ion concentration was recorded for stage 1 and 4 embryos.

Results of one way ANOVA, testing the effect of the factor salinity on calcium ion concentration on *H. crenulatus* embryos at each of 5 stages are shown in the table below.

| Stage | F statistics | *P* |
|---|---|---|
| 1 | $F_{(1), 3, 22} = 40.7$ | 0.000000 |
| 2 | $F_{(1), 3, 32} = 3.4$ | 0.028856 |
| 3 | $F_{(1), 3, 32} = 2.7$ | 0.056960 |
| 4 | $F_{(1), 3, 28} = 5.2$ | 0.005797 |
| 5 | $F_{(1), 3, 16} = 1.4$ | 0.265305 |

See Table 4.5 for Tukey post-hoc comparison of means.

$Mg^{2+}$ concentration

Magnesium concentration decreased with the development of embryo and with the decreasing salinities (Table 4.6). Magnesium was hypo-ionic throughout development in 100% seawater. Hyper-ionic regulation of magnesium at all salinities was observed for stage 1 embryos (significantly different @ $P < 0.05$, ANOVA, Tukey post hoc). Hypo and hyper-ionic regulation of magnesium was observed in later developmental stages in dilute seawater.

Results of one way ANOVA, testing the effect of the factor salinity on magnesium ion concentration on *H. crenulatus* embryos at each of 5 stages are shown in the table below.

| Stage | F statistics | *P* |
|---|---|---|
| 1 | $F_{(1), 3, 23} = 112.2$ | 0.000000 |
| 2 | $F_{(1), 3, 24} = 2.1$ | 0.122262 |
| 3 | $F_{(1), 3, 32} = 3.2$ | 0.036194 |
| 4 | $F_{(1), 3, 23} = 4.6$ | 0.011691 |
| 5 | $F_{(1), 3, 15} = 0.9$ | 0.433740 |

See Table 4.6 for Tukey post-hoc comparison of means.

**Table 4.5** Concentration of calcium in different embryonic stages of *Hemigrapsus crenulatus* after 24 h in different salinities at 15 °C. Values are mean ± SEM of five or more replicates. Means with different letter labels (a,b,c &d) are statistically different @ $P < 0.05$.

| Salinity of seawater (%) | Calcium concentration ($mmol.L^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | Medium | Stage 1 | Stage 2 | Stage 3 | Stage 4 | Stage 5 |
| 100% | 10.7 | 7.36 ± 0.69 [a] | 8.69 ± 0.27 | 8.26 ± 0.44 | 8.27 ± 0.3 [a] | 8.65 ± 0.90 |
| 50% | 5.32 | 4.89 ± 0.51 [b] | 8.70 ± 0.40 | 7.53 ± 0.38 | 6.52 ± 0.18 [b] | 7.45 ± 0.96 |
| 10% | 1.06 | 1.44 ± 0.07 [c] | 7.34 ± 0.18 | 6.95 ± 0.34 | 7.84 ± 0.42 [a] | 6.76 ± 0.38 |
| 1% | 0.11 | 0.94 ± 0.1 [d] | 7.54 ± 0.24 | 6.88 ± 0.34 | 7.47 ± 0.35 [a] | 6.87 ± 0.40 |

**Table 4.6** Concentration of magnesium in different embryonic stages of *Hemigrapsus crenulatus* after 24 h in different salinities at 15 °C. Values are mean ± SEM of five or more replicates. Means with different letter labels (a,b,c &d) are statistically different @ $P < 0.05$.

| Salinity of seawater (%) | Magnesium concentration ($mmol.L^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | Medium | Stage 1 | Stage 2 | Stage 3 | Stage 4 | Stage 5 |
| 100% | 55.8 | 59.44 ± 4.0 [a] | 43.95 ± 1.82 | 22.65 ± 1.61 [a] | 27.93 ± 0.97 [a] | 21.88 ± 1.83 |
| 50% | 27.88 | 36.30 ± 1.64 [b] | 42.94 ± 1.67 | 26.70 ± 0.68 [b] | 25.04 ± 1.21 [a] | 19.63 ± 2.36 |
| 10% | 5.57 | 12.02 ± 1.26 [c] | 39.27 ± 0.99 | 23.39 ± 0.78 [a] | 26.17 ± 1.58 [a] | 19.92 ± 0.47 |
| 1% | 0.56 | 4.19 ± 0.38 [d] | 39.57 ± 1.82 | 24.03 ± 0.42 [a] | 22.36 ± 0.79 [b] | 19.48 ± 0.96 |

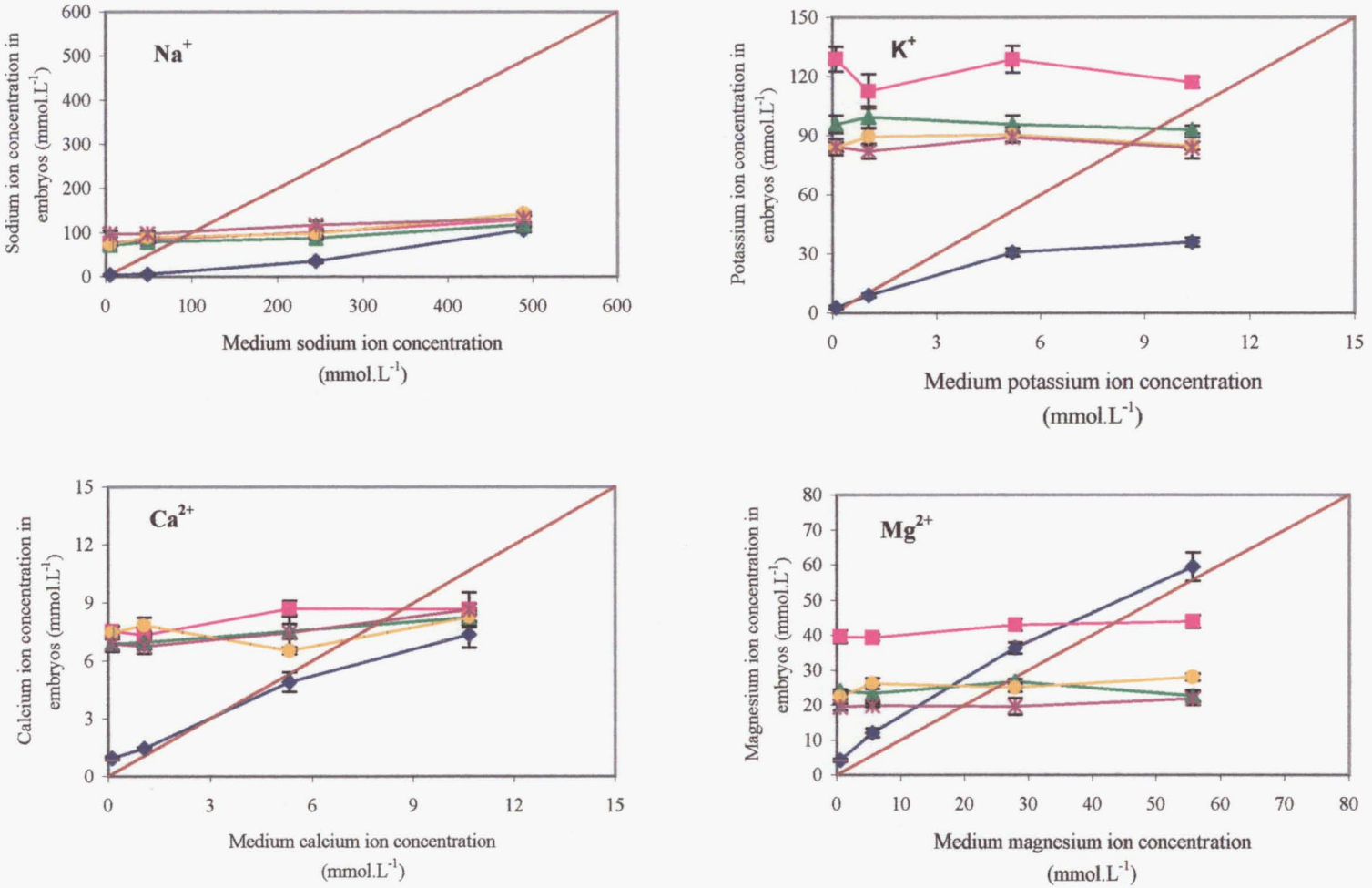



**Fig 4.3** Four cation concentrations in the embryos of *Hemigrapsus crenulatus* as a function of medium ion concentration for: ♦ Stage 1; ■ Stage 2; ▲ Stage 3; ● Stage 4 ✳ Stage 5 and —— isoionic, after 24 h in different salinities. Values are Mean ± SEM.

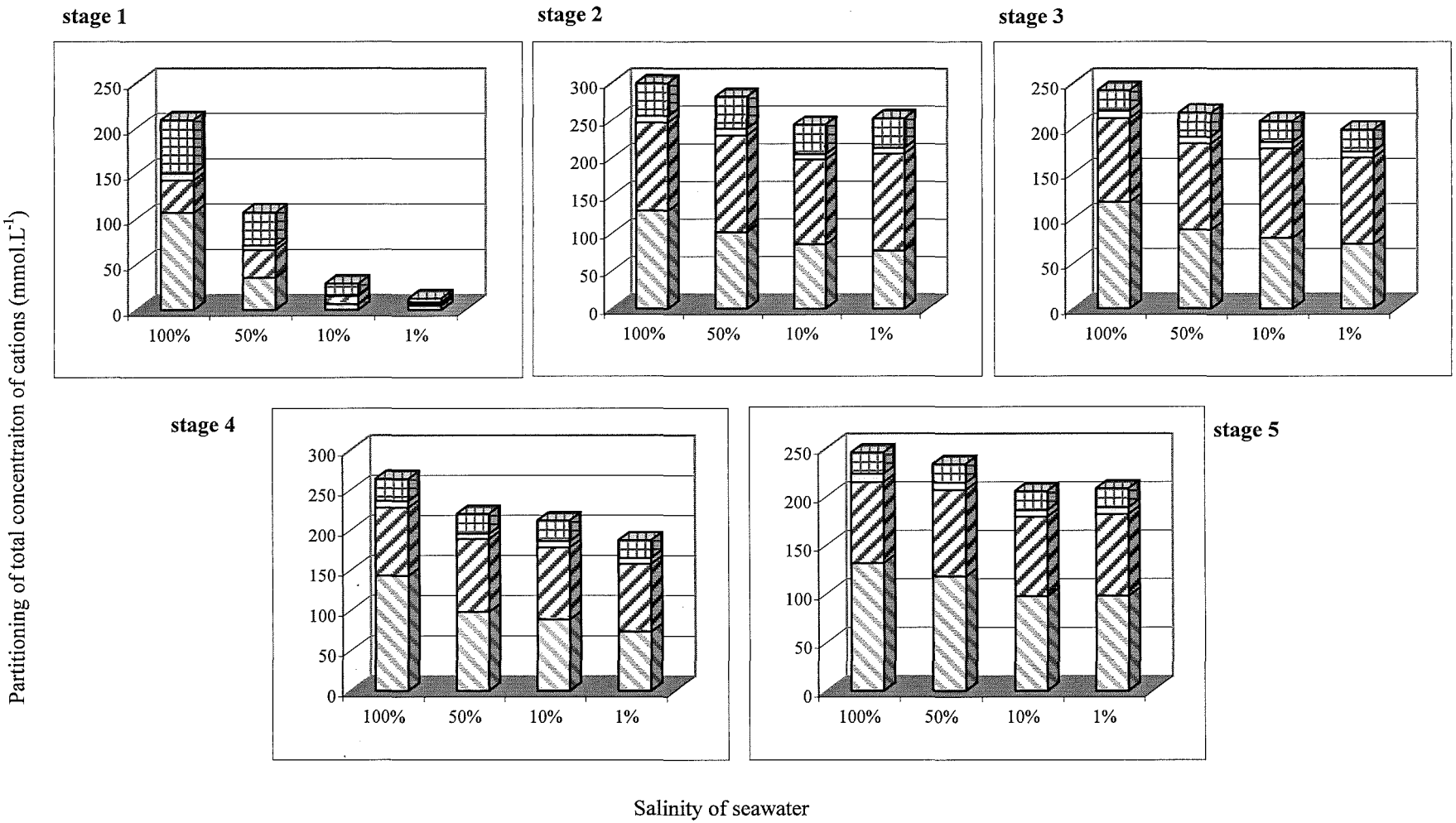



**Figure 4.4** Partitioning of total concentration of cations in the embryos of *H. crenulatus* at different developmental stages after 24 h in different salinities at 15 °C. (▨ Sodium; ▨ Potassium; □ Calcium & ▦ Magnesium ions).

**Contribution of electrolytes to osmolality change in the embryos of *H. crenulatus***

Figure 4.5 shows the estimated osmotic component of the major electrolytes in the embryos of *H. crenulatus* in relation to changes in their measured osmolality, caused by exposure to dilute seawater. In all stages and at all salinities, there was a deficit of several hundred mosmoles in the electrolye component. For pregastrula and gastrula stages embryos, electrolytes and total osmolality changed in parallel. However, for postgastrula stage embryos, the decrease in embryo osmolarity in dilute seawater was accompanied by a much lower rate of change in electrolyes.

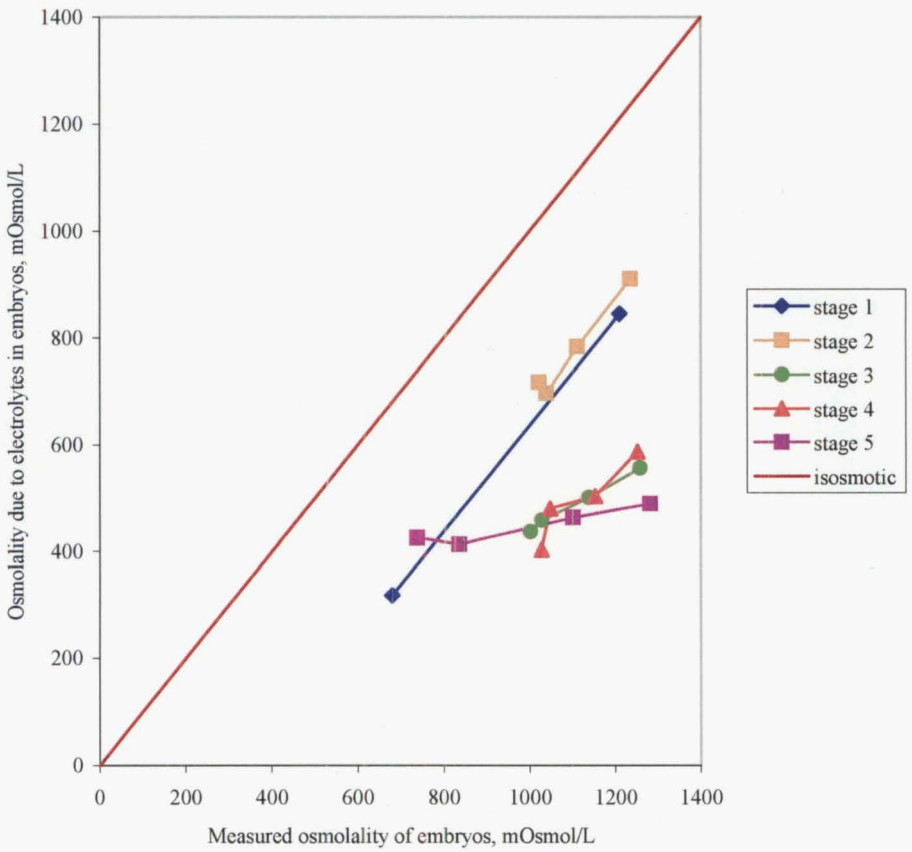



**Figure 4.5** Contribution of electrolytes to osmolality change in the embryos of *H. crenulatus* at different developmental stages at 15 °C. (Uses data from Tables 3.6 to 3.10 and 4.3 to 4.6 and assumes $Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$ were associated with monovalent anions with osmotic coefficient of 0.9, and that the ions were distributed only in the solvent volume).

## DISCUSSION

The overall cation concentration was increased during development in the embryos of *H. crenulatus* and decreased with decreasing salinity. Sodium and potassium were the main cations in these embryos. Rather similar patterns were reported by Leelapiyanaart (1996) for the embryos of *Heterozius rotundifrons*. The measurements of four cations by AAS in developing embryos at different stages after 24 hours exposure to different salinities at 15 $^{0}$C found that postgastrula stage embryos could regulate ions strongly in hyposaline waters. In pregastrula embryos (stage 1) the ion concentrations varied in parallel with the changes in the medium. These changes reflect the changes in total osmolality of these embryos in dilute seawater reported in Chapter 3. The decrease in osmotic pressure may account for by changes in the osmotically active solutes (osmolytes) since the total osmolyte in embryos decreased with osmolality.

It is generally accepted that organisms employ a combination of inorganic ion transport and organic osmolyte (free amino acids) deployment for cell volume regulation (Gilles, 1983; Pierce, 1982, Yancy *et al.*, 1982). For embryos at early and late stages of *H. crenulatus* in 100% seawater, it may be calculated that the contribution to the total osmolality due to sodium, potassium, magnesium and calcium salts decreases from approximately 845 to 489 mOsmol.kg$^{-1}$. This calculation uses solvent volumes from Tables 3.6 to 3.10 and cation concentrations from Tables 4.3 to 4.6 which are assumed to be present as salts of monovalent anions with an osmotic coefficient of 0.9 (see Potts & Parry, 1963). Thus, the remainder of the osmotic pressure of embryos (about 30% and 60% of the total osmotic pressure in early and late stage embryos, Tables 3.6 – 3.10) must be contributed by other osmotically active materials such as free amino acids, yolk products and other organic metabolites. Smaldon (1973) reported a presence of a free amino acid pool in the embryos of *Pisidia longicornis* (Decapoda, Anomura). In these embryos, synthesis of new amino acids during development was observed and suggested that amino acids contribute in the osmoregulatory function.

The contribution of inorganic ions in the osmoregulation in the embryos of *H. crenulatus* was investigated during embryonic development and in relation to salinity stress conditions. Figure 4.5 shows the relative contribution of electrolytes to osmolality change. In *H. crenulatus*, osmolality of embryos at stage 1 decreased from 1210 mOsmol.kg$^{-1}$ in 100% seawater to 680 mOsmol.kg$^{-1}$ (Table 3.6) in 50% seawater after 24 h. The estimated component of the osmolality due to sodium, potassium, magnesium and calcium decreased from 845 mOsmol.kg$^{-1}$ to 318 mOsmol.kg$^{-1}$. This clearly indicates that for stage 1 embryos, changes in internal osmolality with dilution is associated primarily with ion loss. However, for late developing stage embryos the estimated contribution of electrolytes to osmolality change was insignificant, i.e. for stage 5 embryos osmolality decreased from 1283 mOsmol.kg$^{-1}$ in 100% seawater to 1102 mOsmol.kg$^{-1}$ in 50% seawater. The corresponding osmolality contributed by ions decreased from 489 mOsmol.kg$^{-1}$ in 100% seawater to 464 mOsmol.kg$^{-1}$ in 50% seawater. This implies that change in internal osmolality on dilution in these late developing embryos of *H. crenulatus* is associated with loss of other osmolytes, presumably organic solutes.

The important role played by organic osmolytes (free amino acids mostly) has been studied in several cells and tissues of crustaceans in hypo and hyperosmotic stress conditions (Gerard & Gilles, 1972; Gilles, 1977; Tan & Choong, 1981). Gerard & Gilles (1972) found that amino acids play an important role in the isosmotic intracellular regulation process which occurs during acclimation of crab *Callinectes sapidus* from 100% seawater to 50% seawater. He proposed that the intracellular osmotic pressure due to the inorganic ions in muscle tissue of crab is only slightly modified in hyposalinity, thus indicating that they are not involved in volume regulation. Tan (1981) demonstrated the involvement of free amino acids in intracellular osmotic adjustments in *Macrobrachium rosenbergii* to hyperosmotic stress. Amino acid mediated volume regulation has been studied in two molluscan tissues in response to low salinity. In these tissues cell volume regulation is accomplished by an efflux of specific amino acids from the cell (Pierce & Greenberg, 1972, 1973, 1976; Amende & Pierce, 1980). In crab embryos, it is thus possible to suggest that protein, free amino acids and some organic compounds play an important role in egg volume regulation. Alternatively low molecular weight metabolites might

be catabolized or incorporated into macromolecules as suggested by Leelapiyanart (1996).

Individual patterns of regulation for each cation differed at different developmental stages. Sodium concentration was relatively constant in all developing stages. There was a significant increase in the potassium concentration at gastrulation (Table 4.2) which is consistent with extensive cellular proliferation at this time. Magnesium content was significantly decreased with development ($P < 0.05$, ANOVA, Tukey post hoc). A relatively small increase in the calcium ion concentration was observed during development in these embryos. Increase of certain cations with the development of the embryos of teleosts, crab and molluscs has been noted by Hayes et al. (1946); Potts & Rudy (1969) and Shen & Leatherland (1978) Teleost: Leelapiyanart (1996) Crab: Taylor (1977) Molluscs. Brown & Terwilliger (1992) has noted a two fold increase in the hemolymph magnesium concentration in megalopas and 1st and 5$^{th}$ instar juveniles of *Cancer magister* (Dungeness crab) in 100% seawater with that of adults and suggested that this changes may be involved in modulating the oxygen binding properties of hemocyanin.

The average regulation of sodium was hypo- and hyper- for different stages in different salinities (Table 4.3). There was a significant decrease in the sodium ion concentration in hyposaline waters for all developmental stages and it appears to be the major component in osmotic regulation in the different developmental stages. The role played by $Na^+$ in osmo and volume regulation in other tissues and cells has been widely studied. According to Robertson (1970) & Warren and Pierce (1982) sodium plays a major role in *Limulus* myocardial cell volume regulation by decreasing $Na^+$ in hypoosmotically stress conditions. Potts and Rudy (1969) reported that at low concentrations, the relative accumulation of sodium in the perivitelline fluid of salmon embryos is lower and this may be due to the Donnan effect associated with its protein content. Osmoregulation in lobsters is apparently achieved through the hyper-regulation of $Na^+$ (Charmantier *et al.*, 2001). Kalman (1959) inferred that in hyperosmotic saline, sodium concentration remained much lower than in the medium being an active ion in the embryos of trout.

Potassium was always hyperionic in the *H. crenulatus* embryos with that of the ambient seawater. This may reflect the general consideration that potassium is often found in higher concentration in the cells or tissues than in the medium (Mantel & Farmer, 1983). Hyperionic regulation of intracellular potassium in adults of *C. lavauxi* and *Macrobrachium amazonicum* was reported by Leader and Bedford (1978) and Zanders (1992) respectively. The rate of decrease in the potassium ion in low salinities was not significant from post gastrula stage embryos. Loss of potassium as an osmotic solute in hypoosmotic adaptation in many cells and tissues has been demonstrated by Costa *et al* (1980) in *Glycera* coelomocytes; Treherne & Pichon (1978) in *Sabella penicillus*; Willmer (1978) in *Mytilus* cerebro-visceral connective axons. Schmidt-Nielsen (1975) provided data to show that volume regulation in several types of cells exposed to hyperosmotic media was dependent upon $K^+$ and amino acids.

Zander (1992) found that the blood level of calcium in adult *Macrobrachium amazonicum* was regulated hyperionically at all salinities tested. Values (± SE) presented in table 4.5 shows that calcium was hyperionically regulated by post-gastrula stage embryos in low salinities. Similarly, a strong regulation of calcium by the megalopa and adult of *Cancer magister* was demonstrated by Brown *et al* (1992). They found that calcium is strongly hyper-regulated in megalopa and adult hemolymph, as salinity decreases, compared to the first and the fifth instar juveniles. However, changes in the calcium ion concentration in postgastrula stages in hyposaline exposure were insignificant and suggests that calcium is not actively involved in osmotic regulation in these embryos.

Mantel and Farmer (1983) in a review study reported that magnesium is one of the divalent ions strongly regulated under salinity stress conditions in many cells and tissues of crustaceans. Hypoionic regulation of magnesium was demonstrated in the later stage embryos of both *Heterozius rotundifrons* and *Cyclograpsus lavauxi* in dilute seawater (Leelapiyanart, 1996). Engelhardt and Dehnel (1973) stated "hyporegulation of magnesium is the most universal feature of ionic regulation in crustacean blood". It is suggested that the hypotonic regulation of blood magnesium ion concentration is necessary to facilitate neuromuscular impulse transmission, and appears to be a characteristic feature of ion regulation in active decapod crustacea.

However, a notable exception to this general acceptance has seen in this study, as magnesium was hyper-ionically regulated by all developmental stages in 10% and 1% seawater, except in 50% seawater. As Mantel and Farmer (1983) stated, this may partly be due to binding of magnesium to proteins or other ions.

It can be concluded that the four cations $Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$ (and associated anions) were major osmotic effectors in the embryos of *H. crenulatus* but contributed only about half of the total osmolality. The average concentrations of all four cations were better regulated than was total osmolality indicating the involvement of other osmolytes in osmoregulation.

# CHAPTER FIVE

# CHANGES IN $Na^+/K^+$ ATPase ACTIVITY DURING EMBRYONIC DEVELOPMENT OF *HEMIGRAPSUS CRENULATUS*


*Summary*

- There was an increase in the $Na^+/K^+$ ATPase enzyme activity during development of these embryos commencing from gastrula stage.
- This enzyme activity was not detectable in stage 1 embryos (pregastrula).
- The involvement of $Na^+/K^+$ ATPase in osmoregulation was inferred from higher activities of this enzyme measured in stage 4 embryos incubated in 50% seawater.


## INTRODUCTION

Hyper-osmoregulation is accompanied by continuous losses of ions from the internal environment to the external medium. Therefore, mechanisms to replace these ions have to be developed (Lucu, 1990; Mantel & Farmer, 1983). Thus, the demonstrated abilities of the embryos of *H. crenulatus* to survive in dilution and hyper-osmoregulate throughout development (Chapter 3) lead to hypotheses concerning possible physiological mechanisms in osmoregulation in these embryos.

Active ion uptake in animals involves the coordinated function of a number of membrane-associated ion pumps. The most well known transporter is the Na-K pump. The Na-K pump is identified as a membrane-bound ATP-hydrolyzing enzyme system, which is activated by the combined effects of $Na^+$ on the cytoplasmic side and of $K^+$ on the extracellular side of the membrane. Because of its enzymatic properties it is identified as $Na^+/K^+$ ATPase. The enzyme requires $Mg^{++}$ and $Na^+$ and a counter-ion which may be $K^+$ or $NH_4^+$ and is inhibited by ouabain (Pequeux, 1995).

Besides ample evidence in teleosts (Jensen & Madsen, 1998; Jurss *et al.*, 1985; Kelly *et al.*, 1999; Kirschner, 1979; Morgan & Iwama, 1998), evidence for the involvement of $Na^+/K^+$ ATPase in salt uptake by adult and larval crustaceans comes from several sources (Bouaricha *et al.*, 1991; Castilho *et al.*, 2001; Charmantier *et al.*, 2001

review; Flik & Haond, 2000; Harris & Bayliss, 1993; Lee & Watts, 1994; Lima *et al.*, 1997; Lucu & Flik, 1999; Mantel & Farmers, 1983; Morris & Edwards, 1998; Thuet *et al.*, 1988 ; Towle & Kays, 1986; Towle, 1990; Welcomme & Devos, 1988; Zare & Greenaway, 1998). In addition, development of the mechanisms responsible for the strong hypo-osmoregulatory abilities of brine shrimp, *Artemia salina* have found to be linked to high levels of activity of the $Na^+/K^+$ ATPase (Holliday *et al.*, 1990; Lee & Watts, 1994).

The central role played by $Na^+/K^+$ ATPase in salt uptake and elimination by aquatic animals has been inferred primarily from observations of its localization at high specific activities in the epithelia of recognized sodium and chloride regulatory effector organs (osmoregulatory epithelia), notably the gills (Lucu, 1990; Lucu & Flik, 1999; Mantel & Farmer, 1983; Pequeux, 1995; Siebers, *et al.*, 1985; Towle & Kays, 1986; Towle, 1984; Zare & Greenaway, 1998). Castilho *et al.* (2001) in a study with the estuarine crab, *Chasmagnathus granulata*, suggested the presence of different $Na^+/K^+$ ATPase isoforms in anterior and posterior gills with differing affinities for Na, but similar affinity for K, Mg, ATP etc. High specific enzyme activity in the larval salt gland of *Artemia salina* which function as the site of water and salt uptake in these animals, was reported by Ewing & Peterson (1974); Lowy (1984); and Lowy & Conte (1985). Pleopod $Na^+/K^+$ ATPase enzyme activity and osmoregulatory performance were measured in isopods by Holliday (1988).

Another indication of the role of $Na^+/K^+$ ATPase in osmoregulation is the observed increase in the enzyme specific activity following and during acclimation to low salinities. As $Na^+$ gradients increase, additional transport capacity is required to maintain $Na^+$ steady state, since $Na^+$ is a major osmotic effector in the haemolymph of crustaceans (Lucu, 1990; Pequeux, 1995). In some species, during acclimation to low salinities, there appears to be a rapid response (hours) in which it is inferred that activation takes place by "unmasking" of existing $Na^+/K^+$ ATPase enzyme sites during $Na^+$ depletion, and "remasking" with subsequent $Na^+$ loading. In that way the total number of enzyme molecules are thought to remain constant (Holliday, 1985; Savage & Robinson, 1983; Welcomme & Devos, 1988). On the other hand, long-term responses are thought to be based on the synthesis or degradation of $Na^+/K^+$ ATPase molecules, and thus changes in the amounts of the enzyme that are actually present

(Holliday, 1985; Lima *et al.*, 1997; Siebers *et al.*, 1982). Longer term acclimation may also be associated with morphological changes such as membrane amplification (Compére *et al.*, 1989; Taylor & Taylor, 1992).

A number of studies have examined the ontogeny of $Na^+/K^+$ ATPase during development of certain organisms with respect to osmoregulation and metabolism. Leong and Manahan (1997) demonstrated that $Na^+/K^+$ ATPase activity is a significant component of total metabolism during sea urchin development. In the estuarine ghost shrimp *Callianassa jamaicense* (Decapoda) $Na^+/K^+$ ATPase activity increased from early development to pre-hatching stages and appeared to be associated with the hyperosmotic regulatory ability at the time of hatching (Felder *et al.*, 1986). Bouaricha *et al.* (1991) reported that the ATPase activity was variable in the successive stages of larval development of *Penaeus japonicus* and that these variations in $Na^+/K^+$ ATPase activity appear to be correlated with osmoregulation.

Charmantier *et al.* (1998, 2001) noted that an increase $Na^+/K^+$ ATPase activity is probably one of the physiological bases of the changes in patterns of ionic and osmotic regulation at metamorphosis of homarid lobsters. They have previously shown that the osmotic regulation of young postembryonic stages of *Homarus americanus* was affected by the moult cycle (Charmantier *et al.*, 1988).

However, $Na^+/K^+$ ATPase is a ubiquitous transporter involved in cellular as well as whole organism osmoregulation and $Na^+/K^+$ ATPase in ion pumping is a major component of total metabolic rate (Chapter 7: Leong & Manahan, 1997). An increase in total activity is expected during embryonic development. Although there have been several studies on $Na^+/K^+$ ATPase activity in adults and during larval development in crustaceans, there are few studies that have examined this enzyme activity in early developmental stages of crustaceans.

The main objective of this study was to determine the activity of $Na^+/K^+$ ATPase throughout the development of the embryos of *H. crenulatus* and to correlate this with the osmoregulatory ability of the corresponding stages. It is shown that there is a large increase of this $Na^+/K^+$ ATPase enzyme activity during development of these embryos and small increase in the enzyme activity in response to dilution. It is

concluded that there is an integrated series of events linked to the appearance of osmoregulatory epithelia, the increase in $Na^+/K^+$ ATPase activity and to the occurrence of hyper-osmoregulation during development and in response to dilution.

## MATERIALS AND METHODS

### Maintenance of animals

Ovigerous *Hemigrapsus crenulatus* crabs collected from the Avon-Heathcote Estuary were maintained in the aquarium of the Department of Zoology at 15 °C during the spawning period from August to January.

### Experimental media

Seawater at different salinities (1% to 100%) was prepared by diluting "Instant Ocean" salt (Aquarium Systems, inc.) with tap water. Salinity was adjusted using a Wescor 5100 vapour pressure osmometer which had been calibrated with standard solutions of 100, 290 and 1000 $mOsmol.kg^{-1}$. The salinity of the control medium (i.e. 100% seawater) was 35.7 ‰ corresponding to an osmolality of 1050 $mmol.kg^{-1}$.

### Experimental design

Two main protocols were carried out to investigate the activity of $Na^+/K^+$ ATPase with development of embryos and in dilution: "acute" and "long term".

In the acute experiments, groups of eggs at different developmental stages (Chapter 2) were detached from the pleopods of ovigerous crabs taken from the aquarium and exposed to a range of experimental salinity (1%, 10%, 50% and 100% seawater) for 24 h. $Na^+/K^+$ ATPase activity was measured after 24 h. Five replicate groups for each stage and salinity were done.

In the long term experiments, ovigerous crabs carrying stage 2 eggs were reared at 15 °C in 100% and 50% seawater until the eggs developed to stage 5 (close to hatch). Eggs were sampled at intervals when they were at stage 4 and stage 5 in both salinities and the $Na^+/K^+$ ATPase activity was measured. The number of replicates for each stage and salinity was five or eight.

**Tissue acquisition**

Eggs were blotted dry, weighed (about 0.4 g) and homogenized on ice-cold homogenisation buffer containing 250 mmol/L Sorbitol (Sigma Cat. # S-7547), 6 mmol/L EDTA (ethylene diamine tetraacetic acid)(Sigma Cat. # E-1644), 25 mmol/L tris/acetate buffer (Tris[hydroxymethyl] aminomethane) (Sigma Cat. # T-1503), 0.1 mmol/L Dithiothreitol (Sigma Cat. # D-5545) and 0.2 mmol/L PMSF (phenylmethylsulphonyl fluoride)(Sigma Cat. # P-7626) with the pH adjusted to 7.3. Homogenisation was performed with a hand-operated ground glass homogeniser (Wheaton USA 7ml), until no visible eggs were observed. The resulting suspension was centrifuged at low speed (500 g) for 2 min to remove debris. The supernatant was then retained on ice for a short time until $Na^+/K^+$ ATPase activity and protein concentration were measured.

The total number of eggs in each sample was also estimated by counting the eggs in weighed sub-samples from the crab.

**Activity of $Na^+/K^+$ ATPase**

The method used to measure the activity of $Na^+/K^+$ ATPase was adapted from protocols published by Morris & Edwards (1995) and Zare & Greenaway (1998).

Preliminary experiments were performed to determine the optimum ouabain concentration (2 mmol/L or 5 mmol/L) for complete inhibition of $Na^+/K^+$ ATPase activity, optimum temperature (25 °C or 37 °C) and incubation time (5, 10, 20, 30 & 40 min) for the assay. On the basis of these trials, 2 mmol/L ouabain was selected for the inhibition assay buffer and the assay was performed at 25 °C for 20 min incubation time.

*Assay buffers*

Total ATPase activity of the egg homogenate was determined using an assay buffer containing 6 mmol/L $MgCl_2$, 100 mmol/L NaCl, 10 mmol/L KCl and 25 mmol/L

Tris/acetate buffer adjusted to pH 7.3. The second assay buffer lacked potassium and contained 6 mmol/L $MgCl_2$, 110 mmol/L NaCl, 25 mmol/L Tris and additionally 2 mmol/L Ouabain (Sigma Cat. # O-3125) was added and pH was adjusted to 7.3. Thus the second medium assayed the activities of all ATPases except the $Na^+/K^+$ ATPase. The difference in activity of these two assays was taken to represent the activity of $Na^+/K^+$ ATPase. pH adjustments were performed using 10% glacial acetic acid.

*Assay*

The reaction was carried out in 1.5 ml. Eppendorf tubes containing 550 µl of appropriate assay buffers and 25 µl of the tissue homogenate (supernatant) and equilibrated to 25 °C. The assay reaction was initiated by adding 55 µl of 10 mmol/L disodium ATP (Sigma Cat. # A2383) to each tube giving an initial ATP concentration of approximately 1 mmol/L. After 20 min, the reactions were stopped by the addition of 150 µl of 0.6 mol/L TCA (Tri-chloroacetic acid) to each tube. The tubes were then centrifuged for 5 min at 10,000 g and the supernatants were assayed for inorganic phosphate as described below.

**Measurement of phosphate**

Measurement of inorganic phosphate in the supernatant from the assay was made using the method of Peterson (1978).

Supernatant (600 µl) was mixed with 200 µl of acid molybdate (2.5% in 4N HCl)(Sigma Cat. # A-7302), 160 µl of 10% SDS (Sodium Dodecyl Sulfate) and 40 µl of freshly prepared 0.025% ANSA (1-Amino 2-napthol 4-sulfonic acid – Fiske & Subbarow reducer)(Sigma Cat. # 661-8) in 1.5 mL Eppendorf tubes and allowed to stand at about 20 °C (room temperature) for 30 min and absorbances were measured at 700 nm using a Unicam SP 1800 Ultraviolet Spectrophotometer.

The test was routinely calibrated using phosphate standards (di Sodium hydrogen (ortho) phosphate, BDH Analar Prod 10249) in the concentration range 0 to 0.8 $mmol.L^{-1}$.

The total $Na^+/K^+$ ATPase activity was calculated as the ouabain sensitive rate of release of inorganic phosphate ($P_i$) in the presence of ATP per embryo per minute ($pmolP_i$/embryo/min).

**Measurement of protein**

In many previous studies, $Na^+/K^+$ ATPase activity has been expressed as specific activities based on the protein content of the homogenates (Morris & Edwards, 1998; Zare & Greenaway, 1998). To allow comparison with such values, the protein concentration in the homogenate was determined using a test kit (Bio-Rad Protein Assay 500-0006). The test was calibrated using 5 protein standards (BSA) in the range of 0.2 to 0.9 mg/ml.

Homogenate (20 µl) and the standards were pipetted into 1.5 ml Eppendorf tubes and 1.00 ml of diluted dye reagent added to each tube and mixed well. The tubes were incubated at room temperature for 10 min and absorbances were measured at 595 nm using a Unicam SP 1800 Ultraviolet Spectrophotometer.

The specific activity of the $Na^+/K^+$ ATPase was calculated as pmol P released per milligram of egg protein per minute ($pmolP_i$/mg protein/min).

**Data Analysis**

Data are presented as means (± S.E.M.) of five or more replicates. For statistical analysis data obtained for acute experiments were transformed to logarithms. Differences in enzyme activity with development were tested by repeated measures One-way analysis of variance of the transformed data (stage was the categorical

factor), followed by Tukey HSD multiple contrast analysis to identify significant differences among treatments.

Differences in the enzyme activity in the long term experiment were analysed by using a two way factorial ANOVA (between stages and salinity). When significant differences were found, Tukey HSD tests were used for post-hoc comparison of means.

Differences are reported as statistically significant at $P < 0.05$. The program STATISTICA 6 was used for statistical analysis.

## RESULTS

### $Na^+/K^+$ ATPase activity during development of embryos of *H. crenulatus*

The mean values (± S.E.M) of the $Na^+/K^+$ ATPase activity (pmol $P_i$/embryo/min and nmol $P_i$/mg protein/min) and the protein content of single embryo (BSA units, μg) for different developmental stages at 100% seawater are indicated in Table 5.1. No activity was detectable for stage 1 embryos (Cleavage to Blastula). There was a significant increase in the enzyme activity during development ($F_{(1),\,3,\,16} = 332.3$, $P$ = 0.000000, One-way ANOVA; Figure 5.1). ATPase activity per embryo was increased about 15-fold between stage 2 (gastrula stage) and stage 5 (Yolk in 2 lobes) (Table 5.1).

### $Na^+/K^+$ ATPase activity of *H. crenulatus* embryos acutely exposed to hyposaline waters

Changes in the $Na^+/K^+$ ATPase activity of isolated *H. crenulatus* embryos after 24 h in hyposaline waters are shown in Table 5.2 and Figure 5.2. At stages 3, 4, and 5, there was a small (non-significant) increase in the mean enzyme activity in dilute seawater. For gastrulae, a significant two-fold (ANOVA results below and Tukey post hoc contrast analysis; Table 5.2) increase in enzyme activity (pmol $P_i$/embryo/min) was recorded on transfer from 100% seawater to 1% seawater.

Results of one-way ANOVA, testing the effect of the factor salinity on the $Na^+/K^+$ ATPase activity on *H. crenulatus* embryos at each of 4 developmental stages are shown in the table below.

| Stage | F statistics | *P* |
|---|---|---|
| 2 | $F_{(1),\,3,\,12} = 7.73$ | 0.0038 |
| 3 | $F_{(1),\,3,\,16} = 0.37$ | 0.7699 |
| 4 | $F_{(1),\,3,\,16} = 1.87$ | 0.1739 |
| 5 | $F_{(1),\,3,\,16} = 2.94$ | 0.0647 |

Results obtained from the Tukey post hoc test are indicated in the Table 5.2.

**Table 5.1** $Na^+/K^+$ ATPase activity of developing embryos of *H. crenulatus* in 100% seawater at 15 °C. Values are mean ( ± S.E.M.) of 5 replicates.

| Stage of development of embryos | | Protein content of single embryo (BSA units) (μg) | $Na^+/K^+$ ATPase activity per mg protein ( $nmolP_i$/mg / min ) | $Na^+/K^+$ ATPase activity per single embryo ( $pmolP_i$/embryo / min ) |
|---|---|---|---|---|
| **Stage 2** | Gastrula | 2.04 ± 0.2 | 0.16 ± 0.03 | 0.31 ± 0.05 |
| **Stage 3** | Eyespot & chromatophores | 1.02 ± 0.09 | 2.04 ± 0.27 | 2.05 ± 0.28 |
| **Stage 4** | Yolk in 4 lobes | 1.07 ± 0.03 | 10.69 ± 0.67 | 10.76 ± 0.72 |
| **Stage 5** | Yolk in 2 lobes | 1.09 ± 0.08 | 15.57 ± 1.99 | 16.38 ± 0.96 |

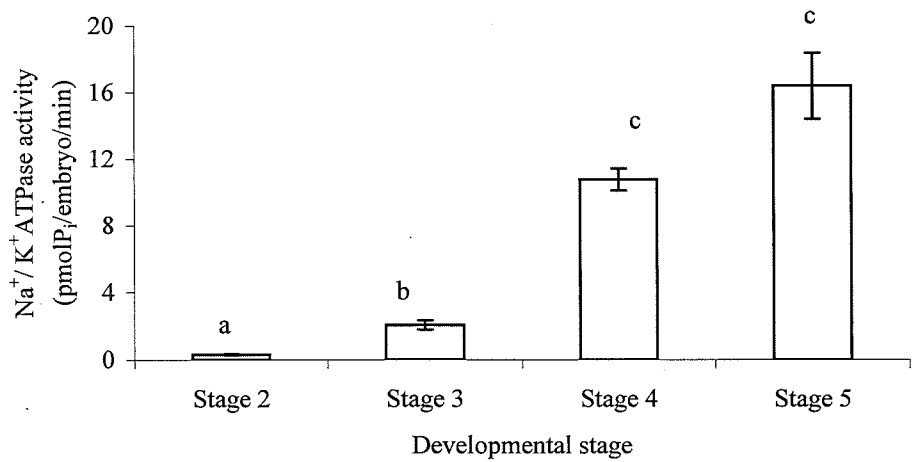



**Fig 5.1** $Na^+/K^+$ ATPase activity of developing embryos of *H. crenulatus* in 100% seawater at 15 °C. (a,b,c) Means with different letter labels are statistically different at $P < 0.05$ ( ANOVA, Tukey HSD).

Table 5.3 indicates the Protein content (BSA units) (µg) of the eggs of *H. crenulatus* at different salinities and stages. Total protein content was halved between gastrula and eyespot stages and thereafter remained more or less constant.

## $Na^+/K^+$ ATPase activity of the embryos of *H. crenulatus* developed in 100% & 50% seawater commencing from stage 2 of development

The $Na^+/K^+$ ATPase activity was also measured for eggs that were incubated by crabs and continuously exposed to either 100% seawater or 50% seawater from the gastrula stage (Figure 5.3 and Table 5.4). As observed in previous experiment, there was a significant increase in $Na^+/K^+$ between stage 4 and 5. At stage 4, the activity of the 50% seawater eggs was double that in 100% seawater (highly significant $P = 0.005$, ANOVA, Tukey HSD test). However, there was no significant difference in $Na^+/K^+$ ATPase activity between 100% and 50% seawater acclimated eggs at stage 5 (pre-hatching).

Results of factorial ANOVA, testing the effect of the factors salinity and stage on the $Na^+/K^+$ ATPase activity on *H. crenulatus* embryos at each of 4 developmental stages are shown in the table below.

| Factor | $F_{(1), 1, 21}$ | *P* |
|---|---|---|
| Stage | 130.7 | 0.000000 |
| Salinity | 3.02 | 0.096435 |
| Stage*Salinity | 11.79 | 0.002489 |

Results obtained from the Tukey post hoc test are indicated in the Figure 5.3

**Table 5.2** $Na^+/K^+$ ATPase activity per embryo ($pmolP_i$/egg/min) of *H. crenulatus* at different stages separated from the crabs and acutely exposed (24 h, 15 °C) to different salinities. Values are Mean (± S.E.M.) of 5 replicates. * significantly different from 100% seawater at $P < 0.01$.

| | $Na^+/K^+$ ATPase activity per single embryo ($pmolP_i$/embryo/min) at different stages | | | |
|---|---|---|---|---|
| Salinity of seawater | Gastrula | Eyespot & Chromatophores | Yolk in 4 lobes | Yolk in 2 lobes |
| 100% sea water | 0.31 ± 0.05 | 2.05 ± 0.28 | 10.76 ± 0.72 | 16.38 ± 0.96 |
| 50% sea water | 0.72 ± 0.29* | 2.23 ± 0.41 | 9.04 ± 0.83 | 18.92 ± 0.67 |
| 10% sea water | 0.70 ± 0.32* | 2.02 ± 0.29 | 11.72 ± 1.16 | 19.37 ± 1.04 |
| 1% sea water | 0.78 ± 0.39* | 2.54 ± 0.42 | 11.02 ± 0.57 | 20.15 ± 1.28 |

**Table 5.3** Protein content (BSA Units -µg) of embryos of *H. crenulatus* isolated at different salinities and stages for 24 h at 15 ° C. Values are Mean (± SEM) of 5 replicates.

| | Protein content per single embryo (BSA units)(µg) at different stages | | | |
|---|---|---|---|---|
| Salinity of seawater | Gastrula | Eyespot & Chromatophores | Yolk in 4 lobes | Yolk in 2 lobes |
| 100% sea water | 2.04 ± 0.20 | 1.02 ± 0.09 | 1.07 ± 0.03 | 1.09 ± 0.08 |
| 50% sea water | 1.66 ± 0.23 | 1.01 ± 0.08 | 1.26 ± 0.09 | 0.94 ± 0.13 |
| 10% sea water | 2.04 ± 0.19 | 1.02 ± 0.15 | 1.25 ± 0.10 | 1.07 ± 0.08 |
| 1% sea water | 1.77 ± 0.23 | 0.99 ± 0.06 | 1.34 ± 0.09 | 0.99 ± 0.10 |

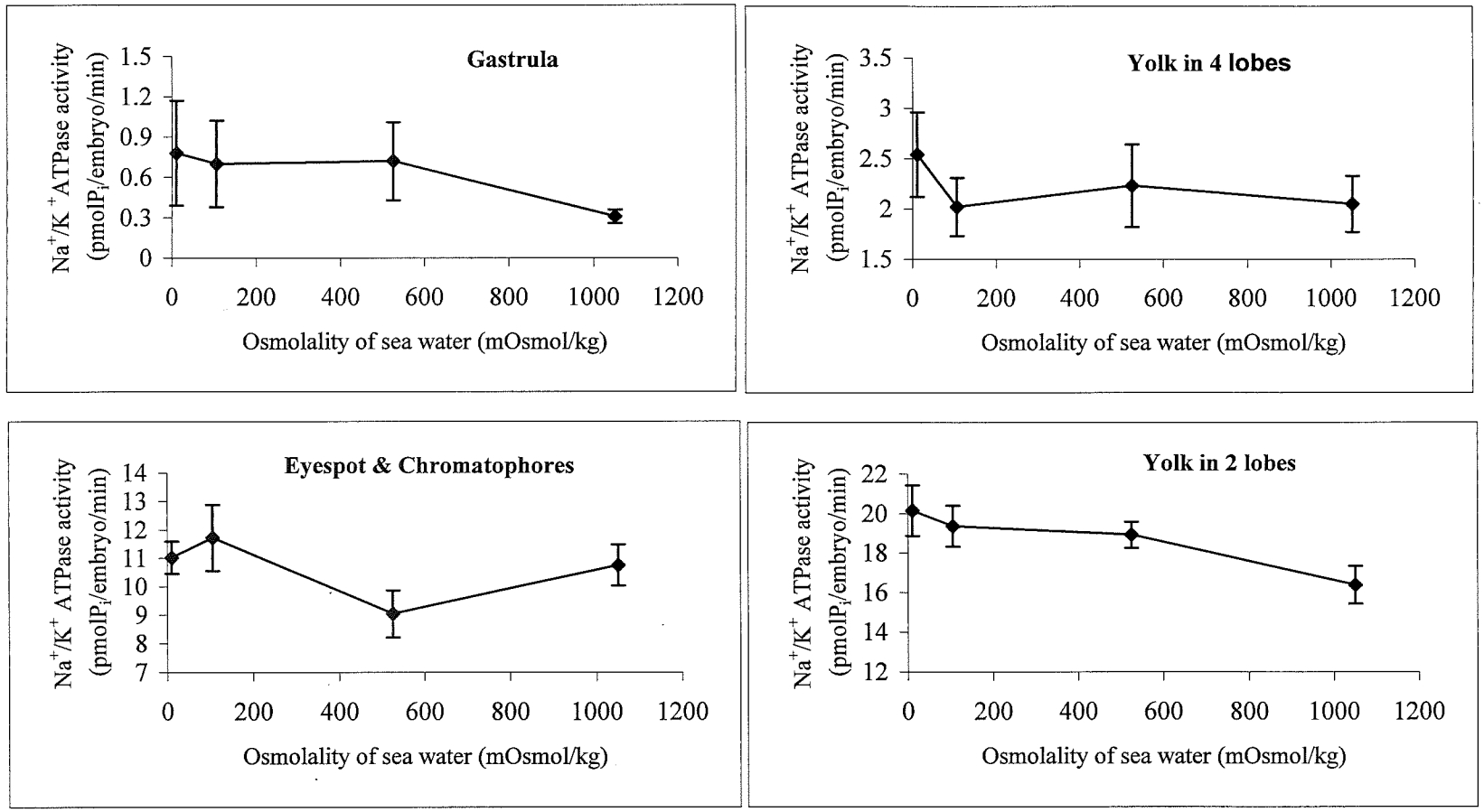



**Figure 5.2** $Na^+/K^+$ ATPase activity of developing embryos of *H. crenulatus* at different stages in different salinities after 24 h at 15 °C. Each point represents the mean value of 5 replicates ± S.E.M..

**Table 5.4** $Na^+/K^+$ ATPase enzyme activity of *H. crenulatus* embryos brooded from the gastrula stage in normal and dilute seawater by crabs in the tidal system up to the yolk in 4 lobe and 2 lobe stages at 15 $^0$C. Values are means ( ± SEM) of 5 replicates. Initial measurements from both groups were made in 100% seawater before placing them into the two treatment tanks.

| Treatment | Protein content of single embryo (BSA units) (µg) | | | $Na^+/K^+$ ATPase activity per single embryo (pmol$P_i$/embryo /min) | | |
|---|---|---|---|---|---|---|
| | Initial (Gastrula) | Yolk in 4 lobes | Yolk in 2 lobes | Initial (Gastrula) | Yolk in 4 lobes | Yolk in 2 lobes |
| Tidal Tank 1 (100% seawater) | 2.01 ± 0.07 | 0.85 ± 0.04 | 0.86 ± 0.03 | 0.5 ± 0.17 | 3.25 ± 0.31 | 12.89 ± 0.39 |
| Tidal Tank 2 (50% seawater) | 2.01 ± 0.07 | 1.09 ± 0.07 | 0.76 ± 0.05 | 0.5 ± 0.17 | 6.61 ± 0.6 | 11.79 ± 0.84 |

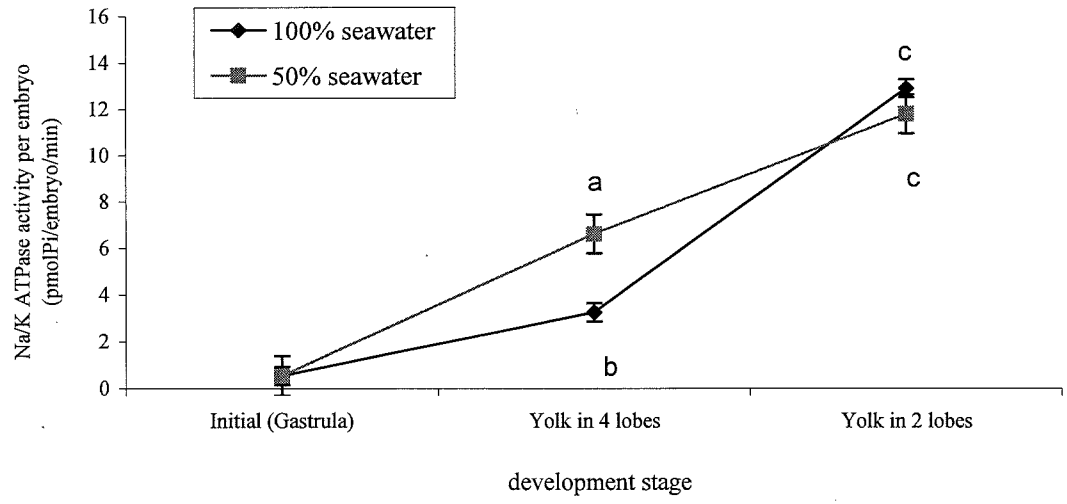



**Figure 5.3** $Na^+/K^+$ ATPase activity of *H. crenulatus* embryos developed from Gastrula stage in the tidal tank system at 15 ° C. (a, b, c) Means with different letter labels were significantly different at $P < 0.05$.

## DISCUSSION

The critical issue addressed in the present study was the involvement of $Na^+/K^+$ ATPase enzyme in hyperosmoregulation in the embryos of *H. crenulatus.* An ontogenetic change in the $Na^+/K^+$ ATPase activity has been demonstrated in these embryos.

The enzyme activity was not detectable for cleavage/blastula (Stage 1) embryos. It was found that there was a significant increase in the ATPase activity during development commencing from the gastrula stage to Yolk in 2 lobe stage in these embryos (Table 5.1 and Figure 5.1). There was a similar increase in the enzyme activity in the embryos that were incubated by crabs and continuously exposed to either 100% or 50% seawater from the gastrula stage (Figure 5.3).

Similarly, an early ontogenetic change in the $Na^+/K^+$ ATPase activity was recorded by Leong & Manahan (1997) for sea urchin embryos. This was described as a step function with near-constant activities from the unfertilized egg to the hatching blastula followed by a rapid increase to the early gastrula. Mitsunaga-Nakatsubo *et al.* (1992) demonstrated similar results for sea urchin embryos and also suggested that increase in total $Na^+/K^+$ ATPase occurred between blastula and gastrulae. Peterson *et al.* (1978) demonstrated in *Artemia salina*, a large increase in the activity of the enzyme after 8 – 12 h hydration of the encapsulated gastrula.

The increase in enzyme activity during early development of the embryos of *H. crenulatus* could possibly be related to the synthesis of the level of enzyme necessary for a successful transition to an osmoregulatory state. It has been shown previously (chapter 3) that the gastrula stage is considered to be the critical stage in these embryos for the development of the capacity to osmoregulate. This increase in enzyme activity is therefore consistent with the involvement of $Na^+/K^+$ ATPase in osmoregulation. A few other species of crustaceans, in which the ontogeny of osmoregulation and the involvement of ATPase enzyme in osmoregulation have been studied, are available for comparative purposes (Bouricha *et al.*, 1991: Charmantier *et al.*, 2001 review; Felder *et al.*, 1986; Lee & Watts, 1994 and Thuet *et al.*, 1988).

Bouaricha *et al* (1991) studied the enzyme activity with larvae and adults of *Penaeus japonicus* and reported that the $Na^+/K^+$ ATPase activity increased in the successive developmental stages, i.e. from zero in nauplii, the activity slightly increased in zoea, and rose sharply in mysis stages 2 and 3. $Na^+/K^+$ ATPase been strongly implicated in sodium transport in brine shrimp *Artemia franciscana* (Lee & Watts, 1994). In *Artemia franciscana*, the activity of this enzyme was first detected in the early prenaupliar stage, increases through the late prenaupliar stage, and continues to increase to high levels in stage 1 nauplii. From the available examples, it can be said that $Na^+/K^+$ ATPase activity is present only in the species which are submitted to high or low or variable salinities (Bouaricha *et al.*, 1991).

It has been hypothesised that the differentiation of ion-transporting and other osmoregulatory structures is correlated with the ontogeny of osmoregulatory mechanisms (Bouaricha *et al.*, 1991; Charmantier, 1998, 2001; Felder *et al.*, 1986; Lee & Watts, 1994). This hypothesis is supported by the morphological observations made on the developing embryos of *H. crenulatus*. Ultrastructure analysis of these embryos (Chapter 2) shows that no such putative osmoregulatory structure detectable in the cleavage/blastula stages (Pregastrula stages). In contrast, a possible ion transporting/permeable area has been demonstrated in all postgastrula stage embryos. The inability of cleavage/blastula stage embryos to osmoregulate and the zero activity of $Na^+/K^+$ ATPase enzyme could possibly reflect the absence of osmoregulatoy epithelium at that stage. It is possible that the silver-staining patch (putative embryonic dorsal organ) is a site of active salt uptake and high $Na^+/K^+$ ATPase activity. However, there is no direct evidence to support this. An alternative hypothesis is that the increased activity of $Na^+/K^+$ ATPase and ion uptake are associated with the general ectoderm and that the stained region is a site of passive ion and water loss.

Evidence can also be gathered from several other sources which support the above hypothesis. Lee and Watts (1994) have suggested that despite the lack of gills, the strong osmoregulatory capacities and high levels of $Na^+/K^+$ ATPase activity in *Artemia* nauplii can be accounted for by the existence of a dorsal organ which function as the osmoregulatory organ in them. In addition, studies done with brine shrimp *Artemia franciscana* by Ewing *et al.* (1974) supported the hypothesis that

occurrence of osmoregulation links to the active involvement of $Na^+/K^+$ ATPase enzyme during development. But note that the dorsal organ in *Artemia* is possibly associated with excretion of ions and has features of ion transporting epithelium. Furthermore, Felder *et al.* (1986) reported the $Na^+/K^+$ ATPase activity of shrimp *Callianassa jamaicense*, which is commonly associated with salt-transporting tissues, increases from early stages of prehatching embryos and reach the highest levels at hatching possibly reflecting the ontogenic development of osmoregulatory abilities of them.

Table 5.2 and Figure 5.2 present the changes in $Na^+/K^+$ ATPase activity of isolated embryos after 24 h acute exposure to hyposaline waters. A significant increase in the enzyme activity in dilution was measured only in gastrula stage embryos. This could be related to the "emergency capacity" of these gastrula stage embryos for sudden short-term reductions in ambient salinity, by synthesising additional enzymes (Harris & Santos, 1993) or activation of existing enzymes.

There was a significant increase in the enzyme activity in the embryos at the 4 lobe stage continuously exposed to dilute seawater (50% seawater) during development (Figure 5.3). This could be interpreted as due to the unmasking of existing enzyme sites at first as a response to sudden reduction of the salinity, followed by the synthesis of additional enzyme to maintain hyper-osmotic conditions effectively. The reason for the lack of difference by the 2 lobe stage is unclear. Possibly by this stage every resources for protein synthesis are depleted. It is also noteworthy that at this late stage osmoregulatory capacity is reduced (Chapter 3).

In conclusion, it was shown that the activity of $Na^+/K^+$ ATPase enzyme was not detectable in stage 1 embryos and there was a significant increase in the ATPase activity during development commencing from gastrula stage. Significant increase in the activity of this enzyme at the 4 lobe stage in these embryos incubated in 50% seawater supports its involvement in osmoregulation. Rapid activation of this mechanism would certainly be advantageous to these embryos since they are subjected to rapid salinity fluctuations during tidal changes in its estuarine environment.

# CHAPTER SIX

# THE PERMEABILITIES AND TURNOVER RATES OF WATER AND SODIUM OF THE EGGS OF *HEMIGRAPSUS CRENULATUS*

*Summary*

- Short turnover times for sodium and water indicate that the egg membranes are highly permeable to water and ions.
- Permeability of eggs to water and sodium show different trends with development.
- The efflux of water and of sodium resolved into two compartments at all stages; a rapidly exchanging pool and a slowly exchanging pool.
- The rapidly exchanging pool is considered to represent the perivitelline space and the slowly exchanging pool the embryo/yolk.

## INTRODUCTION

Osmoprotection of embryos is accomplished in two main ways. Embryos may be enclosed in more or less tightly closed chambers in which they are bathed by a fluid whose osmolality is controlled by the maternal female. Alternatively, embryos may be enclosed in eggs which are directly exposed to the external environment. In such cases, the egg envelopes may provide some isolation from the osmotic conditions of the environment, although this involves a compromise between impermeability to water and ions required for osmotic protection and the maintenance of essential exchanges concerned with respiration, excretion and growth. *H. crenulatus* eggs develop externally and in Chapters 3 and 4 it was demonstrated that the eggs remain hyperosmotic and hyperionic to the environment throughout development, and during dilution. It is thus important to obtain information concerning the permeability of the egg membranes, and of the embryos themselves, to water and ions.

Mechanisms underlying the osmotic protection of aquatic embryos are currently controversial, for crustaceans as well as for other marine groups such as molluscs and elasmobranchs (Charmantier *et al.*, 2001). Most authors suggest that marine

decapodan embryos are osmoconformers and possess highly impermeable egg membranes (reviewed by Charmantier *et al.* 2001). Several studies support the hypothesis that osmoprotection of embryos could be achieved by having a restricted permeability of the egg envelopes during most of their development (Charmantier, 2001). Charmantier and Aiken (1987) observed that the embryos of American lobsters (*Homarus americanus*) were unable to osmoregulate. At low salinity osmotic water intake was limited and diffusional loss of ions was reduced. They suggested that both of these properties originate from the impermeability of the egg membranes. Yonge (1946) demonstrated that the eggs of *Homarus vulgaris* possess two non-living membranes. The outer membrane was considered to have properties identical to those of superficial cuticle of the integument (restricted permeability), whereas the inner membrane was thought to be freely permeable, being chitinous. Neither author considered that the possibility that the cuticle or integument of the embryo itself might be a significant permeability barrier.

Pandian (1970) discussed the permeability of egg membranes during development of *Homarus gammarus*, based on observations of changes in salt (ash) and water content. He concluded that the egg envelope was permeable to salts throughout development, but increased its permeability to water as development proceeded. It was suggested that swelling of the eggs prior to hatching could be due to the accumulation of excretory substances and/or secretion of solutes of osmotic value by special gland cells in the developing embryo, or by the action of a special hatching enzyme whose function is the chemical alteration of the egg membrane. De Vries and Forward (1991) likewise hypothesized that increase in the permeability of the envelopes of some crab embryos before hatching might result from the action of proteolytic enzymes. Without quantitative information on the relative permeabilities of the various surface layers to water and solutes, it is difficult to evaluate these hypotheses.

By contrast, Leelapiyanart (1996) demonstrated that the large eggs of *Heterozius rotundirons* are very permeable to water and salt throughout development. Winnicki and Slomianko (1970) also inferred that throughout embryonic growth of the crab *Rhithropanopeus harrisi*, the egg covering is permeable to water and selectively permeable to salts and suggested that permeability of the egg membranes to different substances varied, depending on their molecular size.

The observation (Chapters 3 and 4) that embryos of both species of *Hemigrapsus* are hyperosmotic and hyperionic to normal and dilute seawater is not easily reconciled with these earlier hypotheses. In principle, there are three possible mechanisms whereby the eggs could maintain a hyperosmotic internal environment. Firstly, the egg could be a closed system with an impermeable barrier to the passage of water and ions across the surface. As mentioned earlier, this seems unlikely because of the requirement for other metabolic exchanges. Moreover, during periods of development extending over many weeks, even an extremely low permeability to water and ions would be associated with steady net loss of salts and/or osmotic water uptake. Secondly, the egg membranes (or the embryos) could be permeable to water but not to salts and other solutes. This would require the development of a high internal hydrostatic pressure (turgor) to oppose osmotic uptake of water. Although the extracellular coats of animal cells may have high mechanical strength, it is unclear that they could support the kinds of hydrostatic pressures required to balance even small differences in osmotic pressure. Thirdly, the egg membranes could be permeable to water and ions and the embryos in a dynamic steady state with a continuous excretion of water (and therefore salts) to balance osmotic uptake and continuous active uptake of salts to balance diffusive and excretory losses, i.e. they actively hyperosmoregulate.

An investigation of the rates of exchange of water and ions with the external medium would help decide among the above alternative explanations for their hyperosmotic state. If turnover times for water and the major ions were found to be significantly shorter than the acclimation period during which hyperosmoregulation was demonstrated (6 – 96 hours) this would eliminate the first and second mechanisms and support active osmoregulation.

Reduced permeability of the body surface to water and to salts are important factors permitting the evolution of euryhalinity because reduced passive exchanges of water and solutes minimise the metabolic cost of osmoregulation (Potts and Parry, 1963; Shaw, 1955; Sutcliffe, 1961). Such costs would be especially acute for *Hemigrapsus crenulatus* which produces very small eggs with a high surface area: volume ratio. Thus a comparison of water and ion permeabilities among different developmental

stages, with *Heterozius rotundifrons* eggs, and with other eggs and organisms would provide an indication of their relative adaptation to hyposaline exposure.

This chapter reports experiments designed to measure the fluxes of water and sodium ions, and permeabilities of the eggs, throughout the development of *H. crenulatus*, based on the washout of the tracers $^{3}H_2O$ and $^{22}Na$. High turnover rates for water and sodium were observed at all developmental stages although there were important changes during development. Equilibration with these tracers provided independent estimates of the water and sodium contents of eggs which are compared with values obtained from morphometry, osmometry and atomic absorption spectroscopy. Compartmental analysis permitted the partitioning of these pools into rapidly and slowly exchanging components.

The term "egg" is used throughout this chapter referring to the embryo plus embryonic membranes.

## MATERIALS AND METHODS

### Maintenance of animals

Ovigerous *Hemigrapsus crenulatus* were collected from Avon-Heathcote estuary during the spawning period and maintained in the aquarium of the Department of Zoology at 15 °C.

### Experimental design

Batches of eggs at each of stages 1 to 5 (as defined in Chapter 2), were detached from ovigerous crabs taken from the aquarium and were equilibrated in either $^{3}H_2O$ labelled seawater or $^{22}Na$ labelled seawater for 24 hours. They were then washed in unlabelled medium for varying periods (0 to 115 min) before measurement of $^{3}H_2O$ and $^{22}Na$ activity remaining in order to determine water and sodium contents, efflux rates and permeabilities. Experiments were carried out at room temperature (~20 °C).

Subsamples of the embryos from the same crab were counted and weighed to provide estimates of single egg mass.

### Water efflux in the eggs of *H. crenulatus*

Groups of 50-100 isolated eggs at the same stage were pooled from 4 - 5 ovigerous crabs, and equilibrated with 2 mL of $^{3}H_2O$ labelled seawater (2 $MBq.mL^{-1}$ = 50 $\mu Ci.mL^{-1}$) in a covered solid watch-glass for 24h. Groups of 4 to 8 eggs were then transferred to ~ 5mL of unlabelled seawater in a larger watch glass for 0, 0.5, 1, 2, 4, 8, 10, 25, 50, 75, 90 or 115 minutes. The watch-glass was agitated gently and the sea water was changed periodically during the washing period. At the end of the time period, eggs were removed on to a filter paper and as soon as adherent fluid had been absorbed (a few seconds) they were transferred to scintillation vials containing 1.0 mL of aqueous scintillation fluid (Beckman MP). For the zero time measurement, the eggs were transferred directly from the labelling medium to the filter paper. The

activity of tritium was measured by liquid scintillation counting (Beckman 5800) and corrected for quenching. Five or more replicate groups of eggs were measured for each developmental stage, at each wash time. The radioactivity of the labelling medium was also measured. Mean egg volume was measured on sub-samples of the same batch using the method described in Chapter 2.

**Sodium efflux in the eggs of *H. crenulatus***

Sodium efflux from *H. crenulatus* eggs was measured using $^{22}Na$ as a tracer. The method was essentially similar to that used for tritium. After estimating the mean egg volume (Chapter 1), eggs were equilibrated to $^{22}Na$-labelled seawater (200 kBq.mL$^{-1}$ = 5 μ Ci.mL$^{-1}$) for 24 hours. Five or more replicates of egg and medium samples were performed for each developmental stage and at each time. The activity of sodium was measured by liquid scintillation counting (Beckman 5800) without quench correction.

**Water and sodium contents**

The total exchangeable water and sodium contents ($C$ nL or nmol, respectively) of single eggs were estimated from their radioactivity at time zero ($A_0$ dpm egg$^{-1}$).

$$C = A_0/A_m$$

where $A_m$ is the radioactivity of the labelling sea water (dpm nL$^{-1}$ or dpm nmol$^{-1}$).

**Compartmental analysis of tracer washout data**

Inspection of plots of the logarithms of egg radioactivity versus time (see Results section) indicated that $^{22}Na$ and $^{3}H_2O$ activities generally did not decline as single exponentials. Thus, the loss in radioactivity of the eggs during washout of $^{22}Na$ and $^{3}H_2O$ were first analysed in terms of biexponential decay curves of the form:

$$A_t = P*\exp(a*t) + Q*\exp(b*t)$$

Where $A_t$ is the total radioactivity remaining at time $t$ (min) (expressed as either nmol $egg^{-1}$ of $^{22}Na$ or nL $egg^{-1}$ of $^3H_2O$, at the specific activity of the loading medium), and $P$ and $Q$ represent radioactivities of "fast" and "slow" pools, declining with rate constants of $a$ and $b$ ($min^{-1}$), respectively.

For initial analysis, a non-linear curve fitting routine supplied with the FigP graphing package (Biosoft Inc.) was used. This applies an iterative procedure (Marquardt method) to determine the four parameters ($P$, $Q$, $a$, $b$) which best fit the model. Curves were fitted to the mean data, weighted inversely by their standard errors. Where all four parameters were statistically significant ($P < 0.05$ that they were non-zero), these values are reported, along with standard errors of the estimates and their corresponding half times ($T_{1/2}$ = Ln (0.5)/$a$ or Ln (0.5)/$b$, min).

In many cases, a rapidly exchanging pool was present but its rate constant ($a$) was too high in relation to the sample intervals to be determined by this method. In such cases, $Q$ and $b$ were determined by linear regression of Ln ($At$) versus time when none of the fast pool remained (> 2 min for $^3H_2O$, > 25 min for $^{22}Na$). The magnitude of the fast pool ($P$) was then determined by subtraction of $Q$ from the total specific activity at time zero ($C = P + Q$).

**Water and sodium efflux and permeabilities of eggs**

Estimates are reported for the slow pool (assumed to represent the embryo) relative to the surface area of the egg envelope ($A$ $cm^2$), which was assumed spherical, of mean diameter, $d$ cm.

$$A = \pi d^2$$

Efflux of water:

$$J_{OUT\,(w)} = \text{Pool size x Rate constant}$$
$$= Q * b \text{ nL } min^{-1}$$
$$= Q * b * 10^{-6}/60 \text{ } cm^3 s^{-1}$$
$$= Q * b * 10^{-6}/(60 * A) \text{ cm } s^{-1} \text{ (area specific)}$$

Efflux of sodium:

$$J_{OUT\,(Na)} = \text{Rate constant x Pool size}$$
$$= Q * b \text{ nmol min}^{-1}$$
$$= Q * b * 10^{-9} / 60 \text{ mol s}^{-1}$$
$$= Q * b * 10^{-9} / (60*A) \text{ mol s}^{-1} \text{ cm}^{-2} \text{ (area specific)}$$
$$= Q * b * 10^{-6} * 60 / W \text{ mmol kg}^{-1} \text{ h}^{-1} \text{ (mass-specific; } W \text{ is the mass of one egg)}$$

**Permeabilities of the egg membranes to water and sodium**

Diffusive water permeability:

$$P_w = \text{Efflux / (Surface Area * Concentration Difference)}$$
$$= Q * b * 10^{-6} /(60 * A) \text{ cm s}^{-1}$$

Sodium permeabilities refer to the influx of sodium from seawater ([Na] = 0.47 mol $L^{-1}$ = 0.47 * $10^{-3}$ mol $cm^3$) on the assumption that the eggs were in steady state (i.e. Influx, $J_{IN}$ = Efflux, $J_{OUT}$).

$$P_{Na} = \text{Influx / (Surface Area * Concentration Difference)}$$
$$= Q * b * 10^{-6} /(60 * A * 0.47) \text{ cm s}^{-1}$$

# RESULTS

## Exchangeable water content and water efflux

Water content and water turnover rates were determined for egg stages 1 to 5 in tritium labelled 100% seawater. The exchangeable water volume of single eggs increased during development from about 6 nL during cleavage (stage 1) to about 19 nL at the 2-lobe stage (stage 5, pre-hatching)(Table 6.1). Semi-logarithmic plots of efflux data indicated that at stages 1 and 2 (cleavage and gastrula), the exchangeable water comprised a small component that exchanged extremely rapidly ($t_{1/2} < 0.5$ min) and a larger more slowly exchanging pool (Figure 6.1, Table 6.1). The fast pool was not present during stages 3 (eyespot) and 4 (4-lobe stage) but reappeared at stage 5. Significant estimates of the rate constant for exchange of the fast pool were obtained using the non-linear curve fitting method for two of the experimental series (stages 1 and 5) and indicate a more than 10 fold decrease in the turnover of this compartment ($t_{1/2}$ decreased from 0.14 to several minutes). (Table 6.1, Figure 6.2).

In relation to their size, the embryos of *H. crenulatus* were very permeable to water at all stages with half exchange times for the slow pool of the order of an hour or less. Stage 1 embryos had the highest permeability ($13.3 \times 10^{-7}$ cm $s^{-1}$, $t_{1/2} = 16$ min; Table 6.1). Water permeability of the slow compartment decreased and $t_{1/2}$ increased approximately by a factor of three between cleavage and hatching ($5.3 \times 10^{-7}$ cm $s^{-1}$, 43 min, respectively).

## Exchangeable sodium and sodium efflux in the eggs of *H. crenulatus*

Sodium efflux into 100% seawater was measured using $^{22}Na$ as a tracer for eggs of *H. crenulatus* at stages 1 to 5. Sodium washout curves fitted by log-linear regression are shown in Figure 6.3 and those fitted to the biexponential model by the non-linear method are shown in Figure 6.4. Sodium efflux was resolved into two compartments at all stages, i.e. a rapidly exchanging pool and a slowly exchanging pool. Calculated

pool sizes, rate constants, and efflux rates are presented for all stages in Table 6.2. Significant estimates of the rate constants for the fast pool were obtained only for stages 1 and 5 but as for water, indicate a large (30-fold) decrease in turnover rate.

Estimates of the mass-specific sodium efflux and sodium permeability of the embryos (Table 6.2) are based on the slow pool data. The embryos of *H. crenulatus* were very permeable to sodium at all stages of their development, exchanging half of the slow pool (and all of the fast pool) within a few hours. In contrast to water, and the fast sodium pool, sodium efflux from the slow pool (presumably representing the embryo itself) increased 20 fold during development from cleavage to hatching.

The total exchangeable sodium content of individual eggs increased during development from about 1 to 4 nmoles between cleavage and hatching. Table 6.3 compares these values with analyses obtained using atomic absorption spectroscopy (Chapter 4).

**Table 6.1** Exchangeable water contents, rate constants and half times for water turnover, rates of water efflux, and diffusive water permeabilities of *Hemigrapsus crenulatus* eggs in 100% seawater at 20 °C.

| Egg stage | Series | Exchangeable water volume in one egg (nL) | | | Rate constant ($min^{-1}$) | | $T_{1/2}$ (min) | | Water efflux* ($pL.s^{-1}$) | Permeability ($10^{-7}$ $cm.s^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Total | Fast | Slow | Fast | Slow | Fast | Slow | | Fast | Slow |
| **Stage 1** - Cleavage/Blastula | **1** | 5.6 ± 0.2 | 2.3 ± 0.2 | 3.3 ± 0.0 | -5.04 ± 1.514 | -0.044 ± 0.0010 | 0.14 | 15.8 | 2.4 | 10960 | 13.3 |
| | **2** | 5.9 ± 1.2 | | 2.7 ± 0.4 | | -0.033 ± 0.0008 | | 20.8 | 1.5 | | 8.1 |
| **Stage 2** - Gastrula | **1** | 8.6 ± 0.3 | | 7.9 ± 0.2 | | -0.0211 ± 0.0002 | | 32.9 | 2.8 | | 10.1 |
| | **2** | 9.3 ± 2.8 | | 6.3 ± 0.9 | | -0.0267 ± 0.0012 | | 26.0 | 2.8 | | 12.8 |
| **Stage 3** - Eyespot & chromatophores | **1** | 11.7 ± 0.3 | | 11.1 ± 0.5 | | -0.0211 ± 0.0004 | | 32.9 | 3.9 | | 11.3 |
| | **2** | 10.2 ± 0.2 | | 9.4 ± 0.4 | | -0.014 ± 0.0003 | | 49.0 | 2.2 | | 7.9 |
| **Stage 4** -Yolk in 4 lobes | **1** | 12.9 ± 0.3 | | 12.9 ± 0.8 | | -0.015 ± 0.0006 | | 46.3 | 3.2 | | 8.3 |
| | **2** | 9.5 ± 0.2 | | 11.0 ± 0.7 | | -0.009 ± 0.0005 | | 75.1 | 1.7 | | 4.8 |
| **Stage 5** - Yolk in 2 lobes | **1** | 18.7 ± 0.7 | 11.5 ± 1.1 | 7.1 ± 0.9 | -0.30 ± 0.063 | -0.016 ± 0.0002 | 2.31 | 42.8 | 1.9 | 159 | 5.3 |
| | **2** | 16.7 ± 1.9 | 5.8 ± 1.5 | 10.6 ± 1.5 | -0.11 ± 0.039 | -0.014 ± 0.0023 | 6.53 | 48.0 | 2.6 | 30 | 9.4 |

Values (± SE) in each series were estimated from tritiated water efflux curves of pooled eggs from 5 crabs with embryos at the same developmental stage.
Positive and negative standard errors of values derived from antilogarithms were averaged.
* based on slow pool data.
Series 1 & 2 represent two separate sets of experiments.

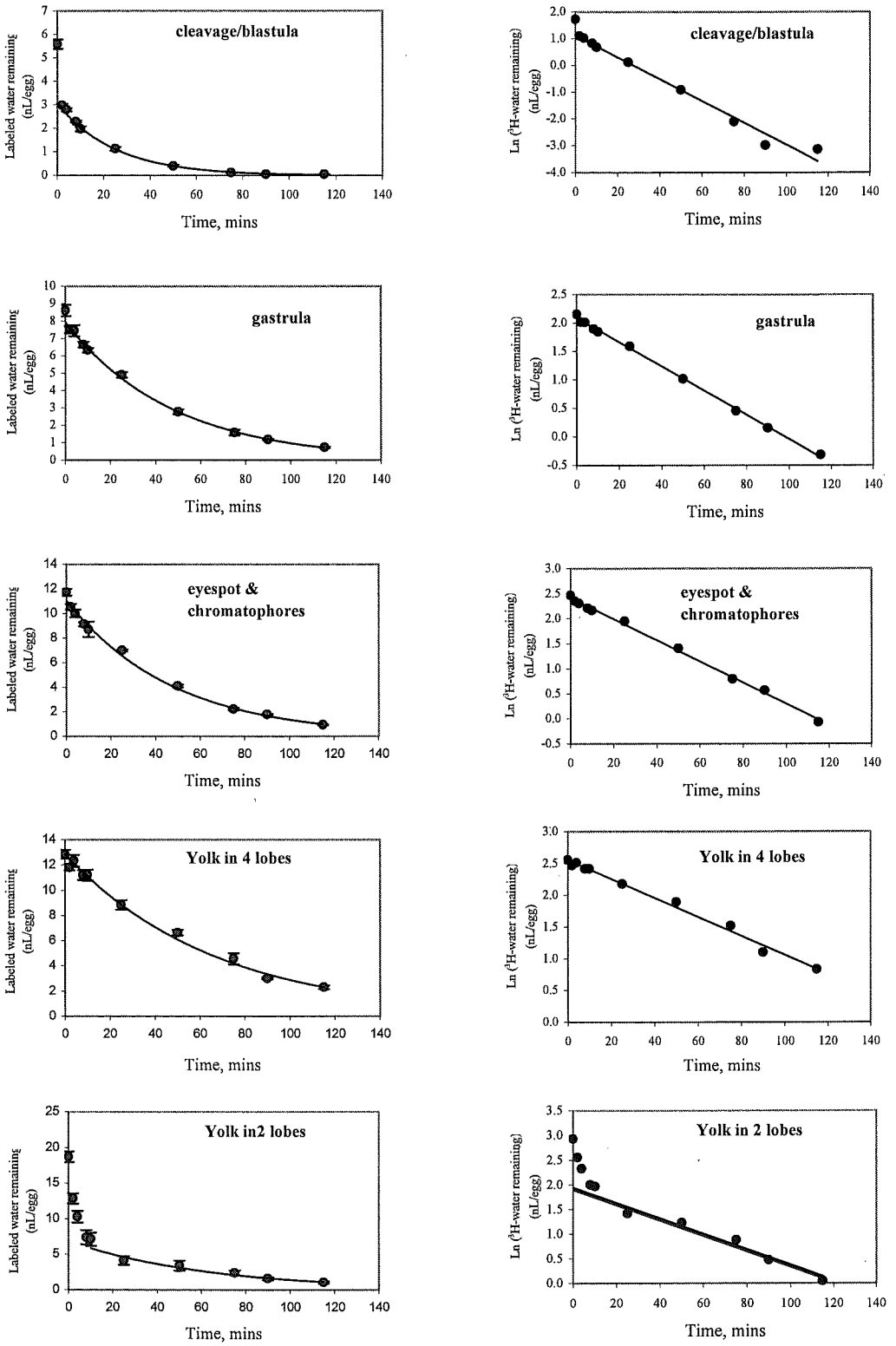
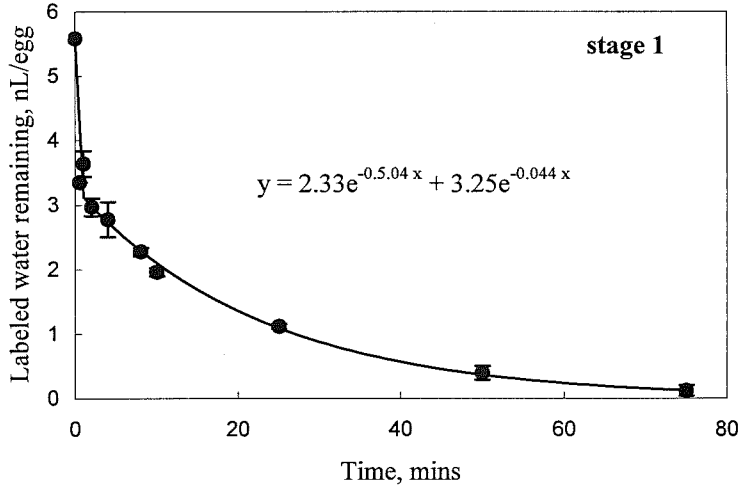
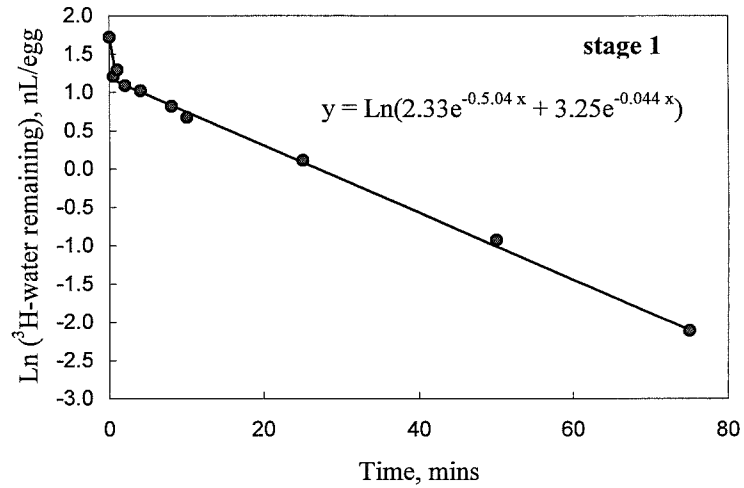
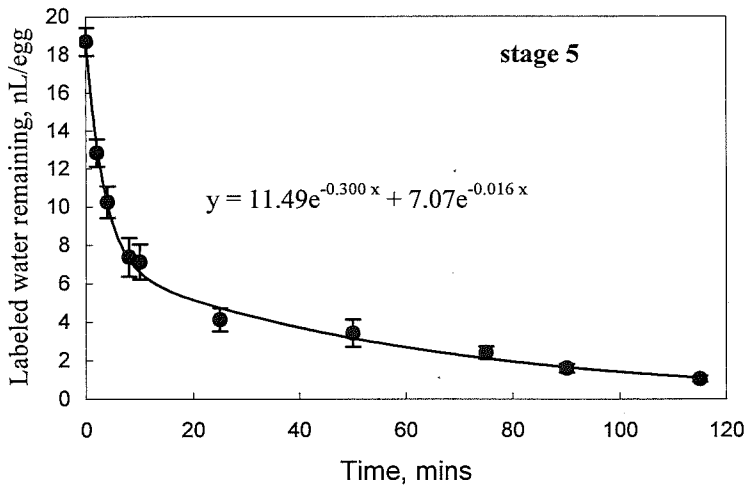



**Figure 6.1** Loss of labeled water by different developmental stages of *Hemigrapsus crenulatus*. Eggs were loaded in tritiated seawater for 24 hours and washed in unlabelled 100% seawater at 20 °C. On the left hand side, data are shown on a linear scale; on the right hand side natural logarithms of the radioactivity are plotted. Each point is the mean of 5 measurements (± SEM on left) on groups of eggs pooled from 5 ovigerous crabs. Trend lines are fitted to the slowly exchanging compartment, assumed to represent the embryo (see also Fig. 6.2 for biexponential fit to cleavage and 2-lobe stages).



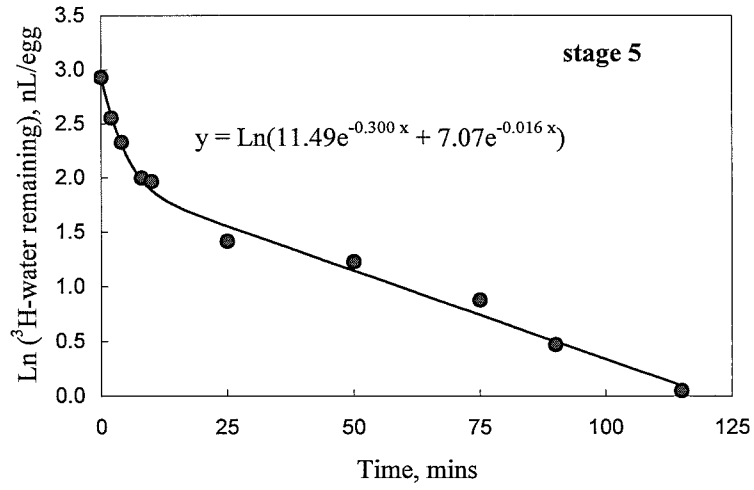




**Figure 6.2** Biexponential curves fitted by non-linear method to $^3H_2O$ efflux in sea water for stage 1 (cleavage/blastula) and stage 5 (yolk in 2 lobes, series 1) eggs of *Hemigrapsus crenulatus* .

**Table 6.2** Exchangeable sodium contents, rate constants and half times for Na turnover, and sodium efflux rates of eggs of *Hemigrapsus crenulatus* at different developmental stages in seawater at 20 °C.

| Egg stage | Series | Mass (µg) | Exchangeable sodium in one egg (nmol) | | | Rate constant ($min^{-1}$) | | $T_{1/2}$ (min) | | Sodium efflux* (pmol $s^{-1}$) | Sodium efflux* (mmol.$kg^{-1}$. $h^{-1}$) | Permeability ($10^{-7}$ cm.$s^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Total | Fast | Slow | Fast | Slow | Fast | Slow | | | Fast | Slow |
| **Stage 1** - Cleavage/blastula | | 7.1 | 1.20 ± 0.06 | 1.0 ± 0.1 | 0.20 ± 0.01 | -2.039 ± 0.188 | -0.003862 ± 0.0006 | 0.3 | 179 | 0.013 | 6.6 | 34.9 | 0.137 |
| **Stage 2** - Gastrula | **1** | 7.8 | 0.86 ± 0.04 | 0.3 | 0.57 ± 0.02 | | -0.0061883 ± 0.0004 | | 112 | 0.059 | 26.9 | | 0.618 |
| | **2** | 9.1 | 0.84 ± 0.05 | 0.5 | 0.35 ± 0.03 | | -0.0063596 ± 0.0011 | | 109 | 0.037 | 14.6 | | 0.352 |
| **Stage 3** - Eyespot & chromatophores | **1** | 13.2 | 1.60 ± 0.11 | 0.4 | 1.18 ± 0.03 | | -0.0034222 ± 0.0003 | | 202 | 0.067 | 18.3 | | 0.503 |
| | **2** | 10.1 | 1.32 ± 0.01 | 0.1 | 1.18 ± 0.05 | | -0.0062436 ± 0.0005 | | 111 | 0.123 | 43.7 | | 1.098 |
| **Stage 4** -Yolk in 4 lobes | **1** | 13.2 | 1.76 ± 0.02 | 0.2 | 1.52 ± 0.02 | | -0.0018525 ± 0.0002 | | 374 | 0.047 | 12.9 | | 0.352 |
| | **2** | 16.3 | 1.72 0.09 | 0.4 | 1.32 ± 0.10 | | -0.0041019 ± 0.0009 | | 169 | 0.090 | 19.9 | | 0.576 |
| **Stage 5** - Yolk in 2 lobes | | 17.4 | 3.93 ± 0.17 | 2.4 ± 0.3 | 1.62 ± 0.3 | -0.076 ± 0.016 | -0.011344 ± 0.0017 | 9.1 | 61 | 0.306 | 63.4 | 18.4 | 1.893 |

Values (± SE) in each series were estimated from $^{22}Na$ efflux curves of pooled eggs from 5 crabs with embryos at the same developmental stage.
Positive and negative standard errors of values derived from antilogarithms were averaged.
* based on slow pool data only.

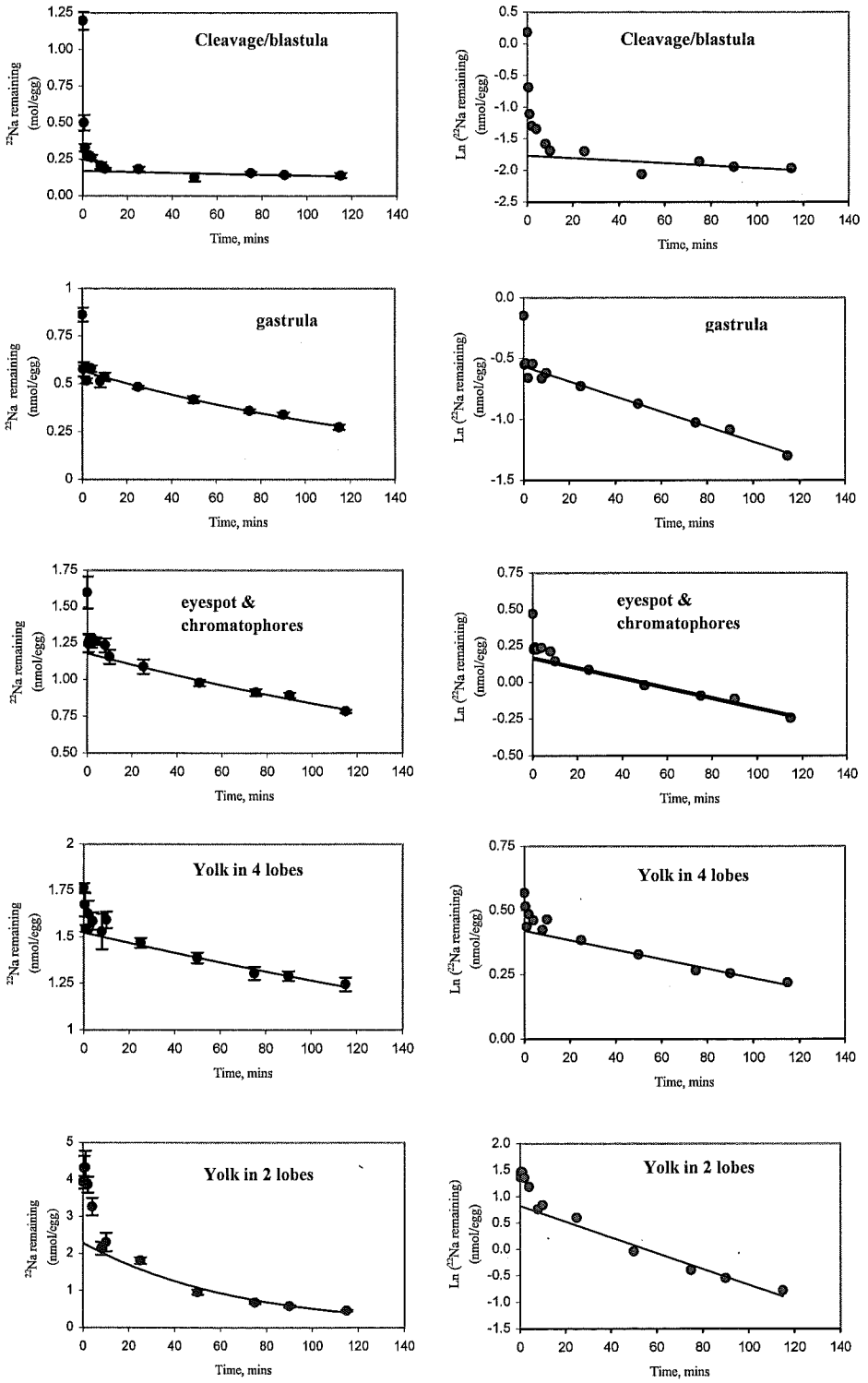



**Figure 6.3** Loss of $^{22}$Na by different developmental stages of *Hemigrapsus crenulatus*. Eggs were loaded in labeled seawater for 24 hours and washed in unlabelled 100% seawater at 20 °C. On the left hand side, data are shown on a linear scale; on the right hand side natural logarithms of the radioactivity are plotted. Each point is the mean of 5 measurements (± SEM on left) on groups of eggs pooled from 5 ovigerous crabs. Trend lines are fitted to the slowly exchanging compartment, assumed to represent the embryo (see also Fig. 6.4 for biexponential fit to cleavage and 2-lobe stages).

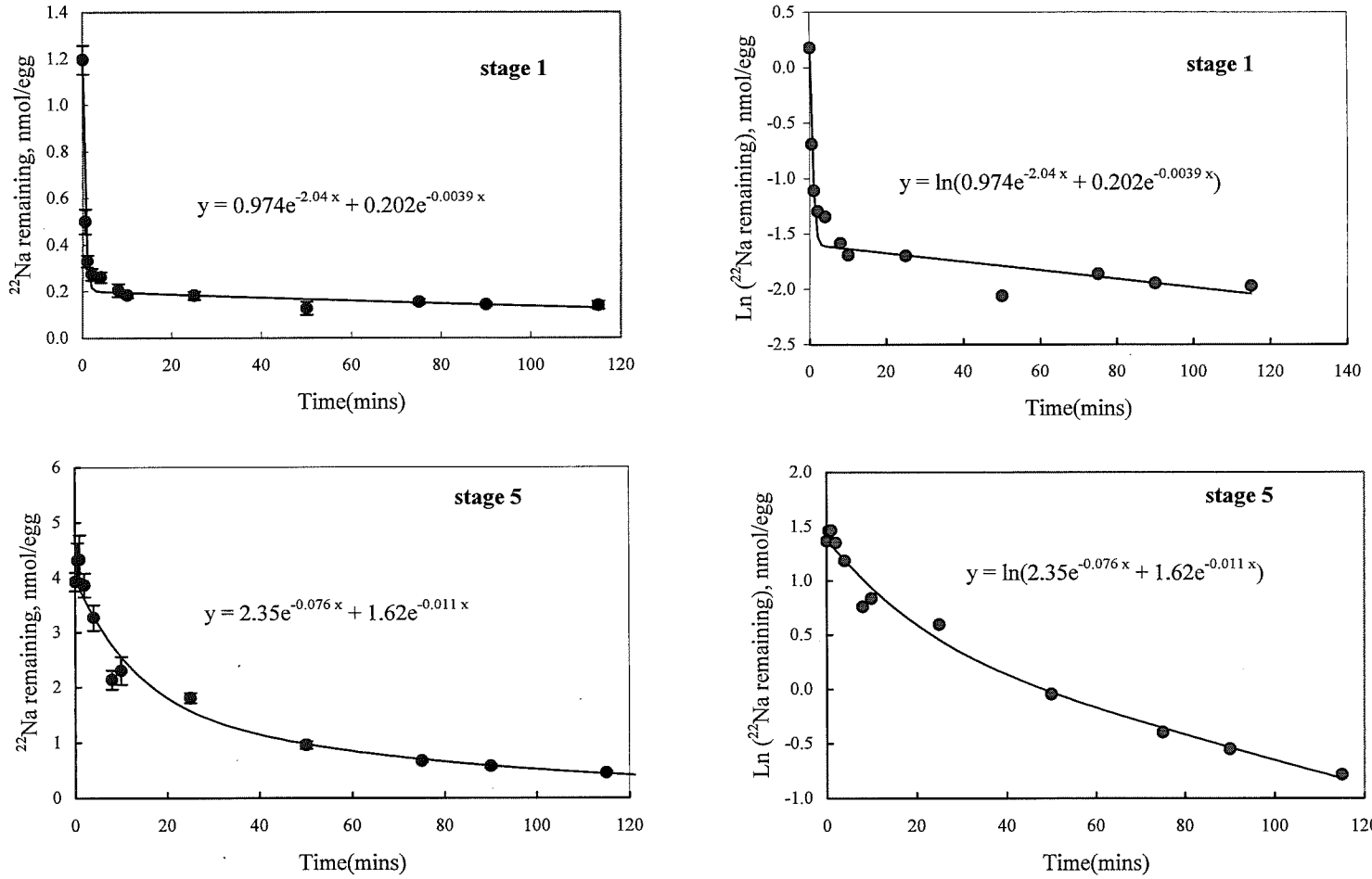



**Figure 6.4** Biexponential curves fitted by non-linear method to $^{22}Na$ efflux in sea water for stage 1 (cleavage/blastula) and stage 5 (yolk in 2 lobes) eggs of *Hemigrapsus crenulatus*.

**Table 6.3** Comparison of AAS* (Atomic Absorption Spectroscopy) and tracer estimates of the sodium content in the eggs of *Hemigrapsus crenulatus* at different developmental stages in 100% seawater. Values are mean ± S.E.M..

| Egg stage | Total Na ($nmol.egg^{-1}$) | | Na Concentration ($mmol.L^{-1}$) | |
|---|---|---|---|---|
| | AAS | $^{22}Na$ | AAS | $^{22}Na$ |
| 1. Cleavage to Blastula | 0.83 ± 0.05 | 1.20 ± 0.06 | 109.49 ± 4.06 | 169.3 ± 8.8 |
| 2. Gastrula | 1.21 ± 0.10 | 0.85 ± 0.03 | 116.93 ± 8.04 | 101.0 ± 5.2 |
| 3. Eyespot & chromatophores | 1.70 ± 0.05 | 1.46 ± 0.07 | 118.87 ± 4.21 | 125.4 ± 4.8 |
| 4. Yolk in 4 lobes | 2.39 ± 0.15 | 1.74 ± 0.04 | 134.58 ± 3.97 | 119.7 ± 3.6 |
| 5. Yolk in 2 lobes | 2.65 ± 0.34 | 3.93 ± 0.17 | 125.28 ± 7.67 | 226.2 ± 9.7 |

- See Chapter 4 for methods. Averaged from two series shown in Tables 4.2 and 4.3.

## DISCUSSION

Turn over rates of water ($^3H_2O$) and sodium ($^{22}Na$) indicated that the egg membranes of *Hemigrapsus crenulatus* are very permeable to water and ions throughout development having half exchange times of the order of few minutes to few hours for water and sodium respectively. Permeability (turnover) of embryos to water and sodium show different trends; i.e. a decrease in diffusive water permeability but an increase in sodium turnover with development of embryos. Presumably this reflects changes of passive properties of the membrane to water while for sodium, it reflects an increase in active uptake. Likewise, Leelapiyanart (1996) studied the permeability of egg membranes of *Heterozius rotundifrons* in terms of tritium and $^{22}Na$ influx rates. She found that those egg membranes are very permeable to water and salt throughout development, although there were some changes in membrane permeability.

It is found that the exchangeable water comprised a small component that exchanged extremely rapidly (not present during stages 3 and 4 of development) and a larger more slowly exchanging pool. Similarly, sodium efflux was resolved into two compartments at all stages, i.e. a rapidly exchanging pool and a slowly exchanging pool. Ultrastructural studies (Chapter 2) revealed that these embryos possess two distinct membranes; "outer" and "inner", surrounding the egg. Thus, it is possible that exchange with the fast pool occurred through the membrane, between the external medium and the perivitelline space of embryos, or between the external medium and the egg membranes themselves. Exchange with the slow pool may occur through the perivitelline space and the embryo and /or yolk cells.

The exchangeable water volume of single embryos increased during development from about 6 nL during Cleavage/Blastula (Stage 1) to about 19 nL at the 2 lobe stage (stage 5)(Table 6.1), values which are comparable with the total volume of the embryos (and a little larger than the solvent volume; Chapter 3). Leelapiyanart (1996) found a similar correspondence between the exchangeable water volume and increases of total volume and solvent volume of embryos during development (Chapter 3). As noted, there were two pools of exchangeable water. The volume of

the outer pool was less than the inner pool. There is not much change in the size of the outer pool (fast pool) and the size of the inner pool increased during development. These observations provide further evidence the hypotheses that the outer pool represents the perivitelline space and the inner represents the embryo/yolk. Therefore, no such change in the size of the outer pool was expected. The increased size of the inner pool with development could perhaps be related to the disappearance of yolk during development which in turn is replaced by water and the general expansion of the embryo.

The permeability of the fast pool decreased significantly for water from cleavage/blastula (stage 1) stage to yolk in 2 lobes (close to hatch). This decrease is likely to reflect changes in the outer membrane and this observation emphasise the problems of osmoregulation experienced by stage 1 embryos to survive in dilute seawater as described in Chapter 3. Early stage embryos had a small extremely exchanging pool ($t_{1/2}$ < 0.05 min) and was not present during stages 3 and 4 development. The fast pool was reappeared again in embryos at close to hatch (stage 5). This reappearance of the fast pool during the stages close to hatch perhaps represents embryonic water related to the hatching mechanisms (De Vries & Forward, 1991; Davis, 1981; Winnicki & Slomianko, 1970; Pandian, 1970).

Table 6.4 compares water diffusive permeability of *H. crenulatus* with a number of different cells and tissues. When compared with the embryos of *Heterozius rotundifrons*, permeability of *H. crenulatus* to water is low. This low permeability could be regarded as an adaptation to smaller size (i.e. greater surface area: volume) of these embryos or as an adaptation to a greater salinity range encountered in the habitat.

Sodium efflux study showed that total exchangeable sodium content of individual embryos increased during development from about 1 to 4 nmoles between cleavage and hatching (Table 6.2). Relatively comparable concentration of sodium for embryos at stages 2, 3 and 4 was observed with AAS (Table 6.3). The high values estimated for embryos at stage 5 measured in turnover experiment could be possibly due to either the differences in egg batches or variations in the development time within the stage. As noted, there were two pools of exchangeable sodium found in these

embryos. Permeability of the fast pool for sodium decreased whereas for slow pool it fluctuated during development. In contrast to water, and the fast sodium pool, sodium efflux from the slow pool increased 20 fold during development from cleavage to hatching.

**Table 6.4** Comparisons of diffusive water permeability between *H. crenulatus* embryos (means of 2 series) and some other cells, tissues and freshwater invertebrates. (*) Data obtained from Stein (1967), Prosser (1973), and Potts and Parry (1963).

| Cells or tissues or membranes | Water permeability ($cm.sec^{-1}$) | References |
|---|---|---|
| Colloidon membrane | $1.2 \times 10^{-1}$ | Krogh (1939) |
| *Cellophane | $4.0 \times 10^{-4}$ | Durbin (1960) |
| *Lipid | $4.4 \times 10^{-4}$ | Huang & Thompson (1966) |
| *Amoeba | $2.3 \times 10^{-5}$ | Prescott & Zeuthen (1953) |
| **Pelomyxa* | $2.5 \times 10^{-5}$ | Belda (1942) |
| **Zoothamnium* | $3.5 \times 10^{-5}$ | Kitching (1938) |
| *Human (adult) erythrocyte | $5.3 \times 10^{-3}$ | Sidel & Solomon (1957); Paganelli & Solomon (1957) |
| *Human (fetal) erythrocyte | $2.3 \times 10^{-3}$ | Sjolin (1954); Barton & Brown (1964) |
| *Squid axon | $1.4 \times 10^{-4}$ | Villegas & Villegas (1960) |
| *Crab muscle | $1.2 \times 10^{-4}$ | Sorenson (1971) |
| *Sialis lutaria* (larva) (Neuroptera) | $5.0 \times 10^{-6}$ | Shaw (1955) |
| *Frog, ovarian egg | $1.28 \times 10^{-4}$ | Prescott & Zeuthen (1953) |
| *Frog, body cavity egg | $7.5 \times 10^{-5}$ | Prescott & Zeuthen (1953) |
| *Pleuronectes* (marine teleost) | | |
| - body cavity egg | $8.6 \times 10^{-6}$ | Potts & Eddy (1973) |
| - shed egg | $1.7 \times 10^{-7}$ | Potts & Eddy (1973) |
| *Zebra fish, ovarian egg | $6.8 \times 10^{-5}$ | Prescott & Zeuthen (1953) |
| *Zebra fish, shed egg | $3.6 \times 10^{-5}$ | Prescott & Zeuthen (1953) |
| *H. rotundifrons* embryos (stage 2) | $2.48 \times 10^{-6}$ | Leelapiyanart (1996) |
| *H. rotundifrons* embryos (stage 3) | $2.92 \times 10^{-6}$ | Leelapiyanart (1996) |
| *H. rotundifrons* embryos (stage 4) | $2.33 \times 10^{-6}$ | Leelapiyanart (1996) |
| *H. rotundifrons* embryos (stage 5A) | $2.33 \times 10^{-6}$ | Leelapiyanart (1996) |
| *H. rotundifrons* embryos (stage 5C) | $2.59 \times 10^{-6}$ | Leelapiyanart (1996) |
| *H. crenulatus* egg (stage 1) | $10.7 \times 10^{-7}$ | Present study |
| *H. crenulatus* egg (stage 2) | $11.45 \times 10^{-7}$ | Present study |
| *H. crenulatus* egg (stage 3) | $9.6 \times 10^{-7}$ | Present study |
| *H. crenulatus* egg (stage 4) | $6.55 \times 10^{-7}$ | Present study |
| *H. crenulatus* egg (stage 5) | $7.36 \times 10^{-7}$ | Present study |

Since the slow pool corresponds to the embryo, this clearly indicates active uptake of sodium by the embryo during development.

A remarkable increase in the sodium uptake was noticed by the gastrula stage. Osmoregulatory (Chapter 3) and ultrastructural (Chapter 2) studies of these embryos demonstrated the appearance of a putative osmoregulatory epithelium by the gastrula stage. Therefore, it is possible to relate this feature with the increased sodium turnover of the embryo on the onset of osmoregulatory abilities. This hypothesis is further supported with the observations made on the $Na^+/K^+$ ATPase activity in these embryos (Chapter 5). This enzyme activity was detectable from gastrula stage and it increased significantly throughout development. Fluctuations in the sodium turnover rates suggest that the embryonic integument may act as a permeability barrier to ions, as well as to water during development. However, the location of $Na^+/K^+$ ATPase in the silver stained patch (presumptive osmoregulatory epithelium, Chapter 2) was not studied in the present study.

A comparison of the sodium turnover rates and permeability between *H. crenulatus* embryos and several other cells, tissues and adult invertebrates are given in the Table 6.5. When compared with other embryo cells (*Heterozius rotundifrons*), sodium permeability of *H. crenulatus* embryos is in relatively similar range. Higher values for the sodium permeability presumably indicate the existence of membrane transporters (channels, passive and active carriers) and/or paracellular routes.

The fact that water and ion uptake is controlled by the moulting process of an animal also needs to be considered as it interrupts the normal physiological life. Lockwood and Inman (1973) studied the sodium fluxes in post-moult *Gammarus duebeni* and found that there is a massive increase in the rate of active uptake above the level found in inter-moult animals and sodium uptake was associated with the water uptake at moult. Therefore, observed variations in water and sodium uptake in different series of the same egg stage (Tables 6.1 and 6.2), may related to embryonic moulting. This may perhaps due to the structural and morphogenetic changes of the membrane in different developmental stages.

**Table 6.5** Comparisons of the sodium turnover rates and permeability (means of two series) between *H. crenulatus* embryos and some other cells, tissues and adult invertebrates. (*) Data obtained from Stein (1967), Prosser (1973), and Potts and Parry (1963).

| Cell, tissue or invertebrate animal | Sodium flux ($mmol.kg^{-1}.h^{-1}$) | Permeability ($cm.s^{-1}$) | References |
|---|---|---|---|
| *Frog muscle | 8.7 | | Harris (1950) |
| *Rat muscle | 225 | | Creese (1954) |
| *Human erythrocyte | 7 | | Harris (1960) |
| *Cat nerve | 64 | | Dainty & Krnjevic (1955) |
| *Sialis lutaria* larva | 0.023 | | Shaw (1955) |
| Synthetic lipid bilayer | | $1 \times 10^{-12}$ | Anderson (1978) |
| Flounder red cells | | $0.63 \times 10^{-9}$ | Cala (1974) |
| *Artemia salina* | | $2.8 \times 10^{-5}$ | Smith (1969) |
| *Heterozius rotundifrons* egg | | | |
| - Stage 2 | 8.84 | | Leelapiyanart (1996) |
| - Stage 5B | 5.24 | | Leelapiyanart (1996) |
| - Stage 5D | 20.5 | | Leelapiyanart (1996) |
| *H. crenulatus* egg | | | |
| - Stage 1 | 6.6 | $1.4 \times 10^{-8}$ | Present study |
| - Stage 2 | 20.75 | $4.0 \times 10^{-8}$ | Present study |
| - Stage 3 | 31 | $8.0 \times 10^{-8}$ | Present study |
| - Stage 4 | 16.4 | $4.0 \times 10^{-8}$ | Present study |
| - Stage 5 | 63.4 | $19.0 \times 10^{-8}$ | Present study |

In summary, turnover times for water and sodium were found to be considerably shorter than the acclimation period (6 – 96 hour, Chapter 3) during which hyperosmoregulation was demonstrated. It is concluded that the embryos of *H. crenulatus* must be in a dynamic steady state with respect to sodium and water. As they are hyperosmotic (except stage 1), it follows that a) either the influx or efflux of $Na^+$ (and counter ions) must be by active transport; b) there must be a steady uptake of water across the outer cells of the embryo by osmosis; and c) this water (and inevitably some salts) must leave the embryos by a different route (i.e. it is excreted).

# CHAPTER SEVEN

# OXYGEN CONSUMPTION OF CRAB EMBRYOS

*Summary*

- Oxygen consumption rates of embryos (per embryo and per unit mass) increased during development.
- No consistent pattern of change in metabolic rates was observed on acute exposure of embryos to hyposaline waters.
- The changes in respiration rates possibly reflect the experimental conditions (time isolated of the detached embryos) rather than the salinity.
- The metabolic cost of development in 50% seawater was higher than in normal seawater, mainly due to a longer development time.

## INTRODUCTION

The relation between the metabolic rate of animals and active transport has been extensively studied both in isolated tissues and in whole animals. Since active transport requires energy, it may be related to oxygen consumption. Given that oxygen consumption rates are one method of estimating metabolic rate (Schmidt-Nielsen, 1983), one can expect a change in salinity of the surrounding medium to be reflected in the oxygen consumption of an animal. It is shown that the developing embryos of *Hemigrapsus edwardsii* and *H. crenulatus* can survive in dilute seawater and hyper-osmoregulate throughout development (Chapter 3). Therefore, it could be hypothesized that exposure to reduction in salinity results in an increase in the rate of oxygen consumption, as salinity would be expected to impose a metabolic cost for osmoregulation in these embryos. It is also generally accepted that organisms experiencing physiological stress expend more energy in coping with that stress (Richmond & Woodlin, 1999). In addition, it is known that the metabolic rates can be affected by developmental stage and the amount of metabolically active tissues present at a particular time (Morgan & Iwama, 1998; Swanson, 1996).

In recent years, a broad relation between osmotic regulation and respiration in adults and larval stages of crustaceans has been demonstrated (Aarset & Aunaas 1990 & 1990; Brown & Terwilliger, 1992; Chen & Chia, 1996; Chen & Lin, 1994; Dalla Via, 1987; Dawirs, 1983; Einarson, 1993; Gaudy & Sloane, 1981; Guerin & Stickle, 1997; Jury *et al.*, 1994; Kutty *et al.*, 1971; Lemos & Alvarez, 2001; Mangum & Winkle, 1973; McAllen & Taylor, 2001; Modlin & Froelich, 1997; Naylor & Bennett, 1997; Rosas *et al.*, 1999 & 1999; Schatzlein & Costlow, 1978; Tedengren *et al.*, 1988; Zanders & Rodrigues, 1992). These authors in general highlighted the difficulty in interpreting the changes in metabolic rates that may accompany exposure to different salinities since it is not always possible to separate behavioural responses from physiological responses.

Aspects of embryonic metabolism in crustaceans have been dealt with by Attard & Hudon, 1987; Babu, 1987; Baeza & Fernandez, 2002; Lardies & Wehrtmann, 1996; Naylor & Bennett, 1999; Pandian, 1970; Taylor & Leelapiyanart, 2001; Valdes *et al.*, 1991; Wear, 1974; Wehrtmann & Kattner, 1998; Wheatly, 1981. It was demonstrated by many authors that the rates of oxygen consumption increased with an increasing osmotic difference between the haemolymph and the environmental medium, consistent with higher energy requirements for osmotic and ionic regulation at low salinities (Aarset & Aunaas, 1990, 1990; Einarson, 1993; Rao, 1968). The lowest respiratory rate occurred when the osmotic difference between haemolymph and medium was minimal. This often occurred at the *in situ* salinity experienced by the animals. Reduced effectiveness of physiological processes could also be caused by reduced energy availability (Boeck *et al.*, 2000). However, the energy actually required for osmo- and ionic regulation in crustacean species calculated from thermodynamic considerations, has been claimed to account for only a minor part of the observed increased in the oxygen consumption rate (Dalla Via, 1987; Krogh, 1939; Potts, 1954).

Further, Potts and Parry (1963) noted that excepting migratory animals in which hormones may override the basic metabolic pattern, most of those animals which osmoregulate increase their metabolic rates as the salinity falls. Also, they explained that animals adjust their respiratory rate to a maximum in their normal environment and they respond to any sudden change by a reduction in the metabolic rate. Kinne

(1971) outlined four different types of responses of the oxygen consumption rates for marine and brackish-water invertebrates, within the tolerable range of salinity. In the Type 1, the rate of oxygen consumption may increase in salinity less than the normal range and or decrease in salinity higher than normal; In Type 2, the rate of oxygen consumption may increase in salinities below or above normal salinity range; Type 3, the rate of oxygen consumption may decrease in salinities below or above normal salinity range; and Type 4, the rate of oxygen consumption is not affected by changes in salinity. He further reported that type 1 and 2 are represented largely by euryhaline invertebrates, type 3 by stenohaline forms and type 4 by extremely euryhaline forms.

In crustaceans, large quantities of food are deposited in the eggs by the female prior to oviposition (Babu, 1987; Clarke, 1993; Wehrtmann & Kattner, 1988). The utilization of yolk by the developing embryos is two sided; one is as an energy source, and the other is for tissue and organ formation. As development proceeds, the embryos utilises the yolk material resulting in a decrease in dry weight (Pandian, 1970). The quality and the quantity of the yolk utilized in different groups varies considerably (Babu, 1987).

As noted above, it was shown that the embryos of *H. edwardsii* and *H. crenulatus* can osmoregulate and this obviously improves their survival in dilution (Chapter 3). However, osmoregulation requires metabolic energy and thus the energetic cost of osmoregulation might affect the rate of development or the fitness of larvae on hatching in dilute seawater. Therefore, it is of interest to determine whether there is a measurable change in the metabolic rates in different salinities for these embryos.

Accordingly, the present study was designed to estimate the effect of salinity on the oxygen consumption of developing embryos. Embryos at different developmental stages were exposed to a range of salinities for short (6, 24 and 96 h) and long (from spawning to hatching) periods. It was found that the effect of salinity on the oxygen consumption of embryos varied for each stage and with exposure time. The oxygen consumption of embryos increased during development. The metabolic rates were relatively unaffected by acute exposure to hyposaline waters. The calculated total

cost of development of a single egg in dilute seawater for both species from spawning to hatching was much higher than in seawater due mainly to prolonged development.

## MATERIALS & METHODS

### Maintenance of ovigerous crabs and embryos

Female ovigerous crabs of *Hemigrapsus edwardsii* and *H. crenulatus* were collected from the intertidal zones at Waipara and Avon-Heathcote Estuary respectively during the spawning periods and were transported and maintained in the aquarium of the Department of Zoology at 15 °C.

### Experimental design

Two main protocols were carried out to study the effect of salinity on the oxygen consumption of the developing embryos of *H. edwardsii* and *H. crenulatus*: "acute" and "long term".

In the acute experiment embryos detached at different developmental stages (Chapter 2) from the pleopods of ovigerous crabs taken from the aquarium were exposed to a range of salinities (100% to 1% seawater) for times ranging from 6 to 96h at 15 °C.

In the long term experiments, ovigerous crabs with stage 1 or stage 2 embryos were reared at 15 °C in either 100%, 50% or 25% seawater until the embryos hatched.

### Experimental media

These test media were prepared using "Instant Ocean" salt (Aquarium Systems, Inc.) and tap water. Salinities were adjusted using a Wescor 5100 vapour pressure osmometer which had been calibrated with standard solutions of 100, 290 and 1000 $mOsmol.kg^{-1}$. The salinity of the control medium (i.e. 100% seawater) was 35.7‰ and the osmolality was 1050 $mOsmol.kg^{-1}$.

**Acute effects of salinity change on oxygen consumption**

Embryos at different developmental stages were exposed to four different salinities (100%, 50%, 10% and 1% seawater) for 6, 24 and 96 h. After the exposure time embryos were blotted, weighed and transferred to the respiration cell to measure the oxygen consumption. The weights used were about 0.05 g for early stage embryos and 0.01 g for late stage embryos. Five or more replicates for each stage and salinity were done.

**Effect of continuous exposure to dilute seawater on oxygen consumption**

Female ovigerous crabs collected from the field were numbered for identification and divided into 2 groups according to the developmental stage of the embryos (chapter 2) and placed into the three tidal system tanks at different salinities (100%, 50% and 25% seawater) after taking initial oxygen consumption of measurements of embryos and until the embryos hatched or aborted.

The osmolality of water was checked periodically and adjusted to ± 5 mOsmol.kg $^{-1}$ of the required salinity during the course of the experiment. Water was changed every fortnight and crabs were fed with mussels weekly.

Subsamples of embryos from the same crab were counted and weighed to provide estimates of single egg volume and mass.

Periodically, sampled embryos were removed to measure the oxygen consumption, as described below.

**Measurement of oxygen consumption of embryos**

The oxygen uptake of embryos was measured using a glass respiration cell of volume 1ml, thermostatted with a water jacket at 15 $^{o}$C. An oxygen electrode (IL 1302 connected to OM 200 Oxygen meter) was fitted into the side of the cell. Known

weights of embryos were introduced into the respiration cell through a hole at the top, and the cell closed by a small glass stopper. Gentle mixing was done by a magnetic spin bar within the respiration cell. Oxygen consumption by the embryos produced a decrease in $PO_2$ and the rate of oxygen uptake was calculated using the $PO_2$ change over time for embryos and blank run of similar time. Embryos were allowed to equilibrate for 5 min in the respiration cell before taking measurements. Measurements were taken after 10, 15, 20 and 25 min depending on the stage and salinity. Usual time for the measurements for all developmental stages were 20 min.

To obtain the Mass specific oxygen consumption of embryos ($Mo_2$), the difference in $\Delta Po_2$ between the crab embryos and the blank (obtained from the linear part of the $Po_2$) was corrected using the oxygen solubility coefficients at water bath temperature (15 °C) and by converting the amount of oxygen consumed as follows:

$$Mo_2 = \frac{(\Delta PO_2 \text{ embryos} - \Delta PO_2 \text{ blank}) \times a \times V \times 60}{T \times W} \quad \mu mol.g^{-1}.h^{-1}$$

Where, a is the solubility of oxygen in seawater at the experimental salinity (calculated by interpolation from the values 1.6218 $\mu mol.L^{-1}$ $torr^{-1}$ and 2.0101 $\mu mol.L^{-1}$ $torr^{-1}$ for 100% seawater and freshwater respectively) at water bath temperature 15 °C. V is the volume of the respiration cell (L); T is the time of the experiment (min) and W is the mass of egg sample (g).

**Data Analysis**

Mass specific oxygen consumption of embryos and oxygen consumption of a single egg are expressed as mean ± S.E.M. Differences in the oxygen consumption during development of embryos of both *H. edwardsii* and *H. crenulatus* was tested by one way analysis of variance (categorical predictor (factor) was the stage). Two way analysis of variance was used to test for significance among treatments (salinity and time were the categorical predictors) for each stage. Subsequent multiple comparisons of means were performed using the Tukey HSD post-hoc test.

Statistical significance was accepted at $P < 0.05$. Differences in oxygen consumption rates in the embryos of both species continuously exposed to low salinities were tested on selected days of development using one way analysis of variance (salinity was the categorical predictor). Accepted statistical significance levels are indicated in the result section.

The total cost of development of single embryos was estimated for each species in 100% and 50% seawater at 15 °C. This was calculated by multiplying the mean oxygen consumption of single embryos in each sample interval of the long term experiment by the duration of the interval and then summing these values.

For statistical comparison of the cost of development, 5 independent estimates were obtained for 100% seawater and for 50% seawater embryos by combining single $MO_2$ measurements selected at random at each sample time. These estimates were compared by Student's t-test. Statistical significance was accepted at $P < 0.05$ and $P < 0.02$.

The programme STATISTICA 6 was used for statistical analysis.

## RESULTS

### Oxygen consumption of developing embryos of *Hemigrapsus edwardsii*

Table 7.1 presents the mean values (± S.E.M.) of the mass specific oxygen consumption of embryos ($\mu mol.g^{-1}.h^{-1}$) and oxygen consumption of a single egg ($pmol.embryo^{-1}.h^{-1}$) of *Hemigrapsus edwardsii* at different developmental stages. Oxygen consumption of embryos increased during development. Blastula stage embryos had the lowest respiration rates per gram and per egg (Table 7.1). The rate of increase was greater at the end of the development than at the beginning (Figure 7.1). About 30-fold increase in the oxygen consumption of a single egg was recorded by stage 5. There were significant differences between oxygen consumption of single embryos at each stage of development ($F_{(1),\,4,\,40} = 108.1$, $P = 0.00$, one way ANOVA), except for the first two stages in development. Results obtained from the Tukey Post hoc multiple comparison test are indicated in the Figure 7.1.

### Oxygen consumption of developing embryos of *Hemigrapsus crenulatus*

As for *Hemigrapsus edwardsii*, oxygen consumption of embryos of *H. crenulatus* increased with development (Table 7.2 & Figure 7.2). Blastula stage embryos had the lowest respiration rate and there was about fifteen-fold increase in the oxygen consumption of per embryo by stage 5 (yolk in 2 lobes). Differences between mean values of oxygen consumption per embryo at each stage of development were tested using one way analysis of variance and there were significant differences in the oxygen consumption between each stage of development ($F_{(1),4,48} = 120.2$, $P < 0.00$). Results obtained from the Tukey Post hoc multiple comparison test are indicated in the Figure 7.2.

### Time course of changes in oxygen consumption of embryos of *Hemigrapsus edwardsii* in different salinities

Tables 7.3, 7.4 and 7.5 present the mean values (± S.E.M.) of the mass specific oxygen consumption of embryos ($\mu mol.g^{-1}.h^{-1}$) for different stages and in different

**Table 7.1** Oxygen consumption of developing embryos per unit mass ( μ mol.$g^{-1}$.$h^{-1}$ ) and per embryo ( p mol.$embryo^{-1}$.$h^{-1}$ ) of *Hemigrapsus edwardsii* at different stages of development in 100% sea water (1000 ± 5 mOsmol/kg) at $15^0$ C. Values are mean ± S.E.M.

| Stage of embryos | Weight of one embryo (μg) | $Mo_2$ ( μmol.$g^{-1}$.$h^{-1}$ ) | $Mo_2$ ( p mol.$embryo^{-1}$.$h^{-1}$ ) | n |
|---|---|---|---|---|
| stage 1 (Cleavage to Blastula) | 19.7 ± 0.34 | 1.05 ± 0.10 | 20.7 ± 2.02 | 13 |
| stage 2 (Gastrula) | 22.1 ± 5.49 | 3.73 ± 0.21 | 82.3 ± 4.57 | 8 |
| stage 3 (Eyespot & Chromatopores) | 26.8 ± 0.45 | 5.43 ± 0.14 | 144 ± 3.76 | 8 |
| stage 4 (Yolk in 4 lobes) | 31.0 ± 0.89 | 8.35 ± 1.01 | 262 ± 34.8 | 8 |
| stage 5 (Yolk in 2 lobes) | 36.5 ± 0.57 | 17.13 ± 1.25 | 625 ± 45.6 | 8 |

**Table 7.2** Oxygen consumption of developing embryos per unit mass ( μ mol.$g^{-1}$.$h^{-1}$ ) and per embryo ( p mol.$embryo^{-1}$.$h^{-1}$ ) of *Hemigrapsus crenulatus* at different stages of development in 100% sea water (1000 ± 5 mOsmol/kg) at $15^0$ C. Values are mean ± S.E.M.

| Stage of embryos | Weight of one embryo (μg) | $Mo_2$ ( μmol.$g^{-1}$.$h^{-1}$ ) | $Mo_2$ ( p mol.$embryo^{-1}$.$h^{-1}$ ) | n |
|---|---|---|---|---|
| stage 1 (Cleavage to Blastula) | 7.3 ± 0.09 | 1.94 ± 0.16 | 14.3 ± 1.31 | 13 |
| stage 2 (Gastrula) | 11.2 ± 0.23 | 5.01 ± 0.33 | 55.7 ± 3.44 | 10 |
| stage 3 (Eyespot & Chromatopores) | 12.2 ± 0.20 | 9.47 ± 0.38 | 115 ± 4.17 | 10 |
| stage 4 (Yolk in 4 lobes) | 12.6 ± 0.09 | 10.99 ± 0.39 | 139 ± 5.62 | 10 |
| stage 5 (Yolk in 2 lobes) | 13.4 ± 0.11 | 16.38 ± 0.83 | 219 ± 11.3 | 10 |

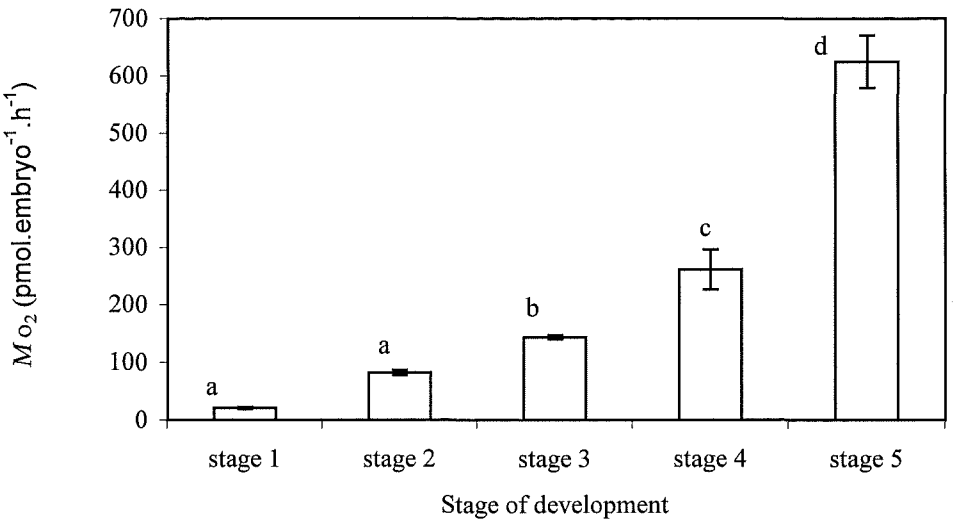



**Figure 7.1** Oxygen consumption per embryo of *Hemigrapsus edwardsii* (pmol.embryo$^{-1}$.h$^{-1}$) at different stages of development in 100% seawater at 15 °C. Values are mean ± S.E.M. Means with different letter labels (a, b, c & d) are significantly different at $P < 0.05$. N values are given in the table.

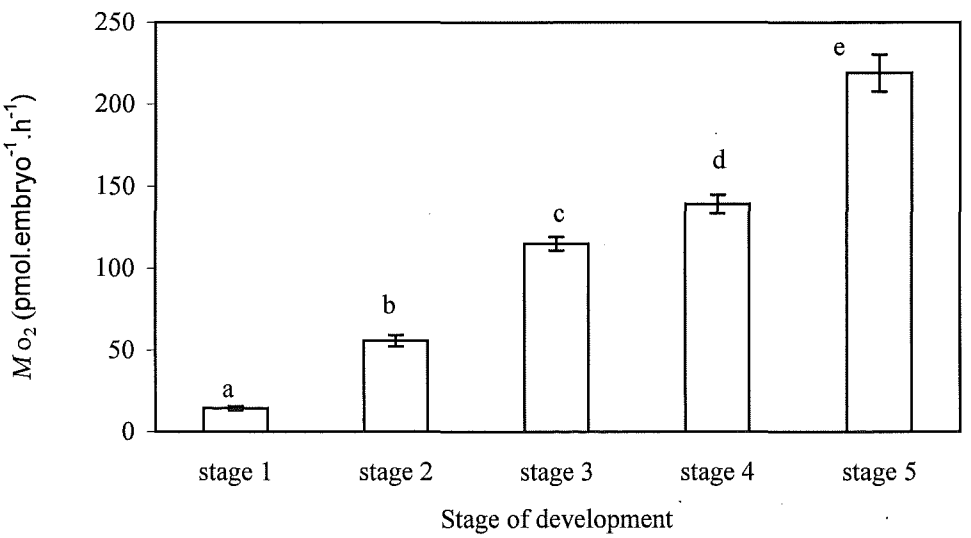



**Figure 7.2** Oxygen consumption per embryo of *Hemigrapsus crenulatus* (pmol.embryo$^{-1}$.h$^{-1}$) at different stages of development in 100% seawater at 15 °C. Values are mean ± S.E.M. Means with different letter labels (a, b, c & d) are significantly different at $P < 0.05$. N values are given in the table.

salinities after 6, 24 and 96 h respectively. $Mo_2$ of embryos generally decreased with dilution and time. However, in stage 1 embryos oxygen consumption in dilute seawater was increased after 96h.

Figure 7.3 shows the oxygen consumption per embryo ($pmol.embryo^{-1}.h^{-1}$) for different developmental stages in different salinities with time. Rates of respiration were different for each stage in different salinities and times. Stages 1, 2 and 4 showed an increase in the rate of respiration in low salinities. However, there was a significant decrease in the rate of respiration in dilute seawater for stages 3 and 5 embryos during 6 to 96 h exposure period. There were significant effects of salinity, time and salinity*time interaction factor on the oxygen consumption of stage 1 embryos (ANOVA, see table below). A significant effect of the exposure times for stages 3, 4 and 5 embryos was recorded (ANOVA, see table below). There was no effect of either salinity or time on the oxygen consumption of stage 2 embryos.

Results of two way ANOVA testing the effect of the factors salinity and time on the oxygen consumption on *H. edwardsii* embryos at each 5 developmental stages are shown in the table below.

| | Salinity | | Time | | Salinity * Time | |
|---|---|---|---|---|---|---|
| | $F_{(1),3,40}$ | *P* | $F_{(1),2,40}$ | *P* | $F_{(1),6,40}$ | *P* |
| Stage 1 | 18.9 | 0.000000 | 54.3 | 0.000000 | 14.7 | 0.000000 |
| Stage 2 | 2.5 | 0.064005 | 2.7 | 0.071653 | 0.3 | 0.938982 |
| Stage 3 | 0.2 | 0.921015 | 9.5 | 0.000226 | 0.3 | 0.934738 |
| Stage 4 | 0.1 | 0.960968 | 71.6 | 0.000000 | 0.4 | 0.867368 |
| Stage 5 | 2.4 | 0.071017 | 34.3 | 0.000000 | 1.7 | 0.125177 |

Figure 7.3 indicates the results obtained from the Tukey Post hoc test .

**Time course of changes in oxygen consumption of embryos of *Hemigrapsus crenulatus* in different salinities**

Mean values (± S.E.M.) of the mass specific oxygen consumption of embryos ($\mu mol.g^{-1}.h^{-1}$) of *H. crenulatus* at different salinities and times are shown in tables 7.6, 7.7 and 7.8. As for *H. edwardsii*, rates of respiration decreased with time

**Table 7.3** Mass specific oxygen consumption of embryos ($\mu mol.g^{-1}h^{-1}$) of *Hemigrapsus edwardsii* in different developmental stages and in different salinities after 6 h at 15 °C. Values are mean ± S.E.M. of five or more replicates.

| Salinity of sea water (%) | $Mo_2$ ($\mu mol.g^{-1}h^{-1}$) | | | | |
|---|---|---|---|---|---|
| | Stage 1 | Stage 2 | Stage 3 | Stage 4 | Stage 5 |
| 100% | 1.19 ± 0.15 | 3.38 ± 0.26 | 4.67 ± 0.34 | 4.90 ± 0.29 | 18.76 ± 1.52 |
| 50% | 1.26 ± 0.16 | 3.69 ± 0.23 | 4.58 ± 0.49 | 4.48 ± 0.5 | 15.66 ± 1.63 |
| 10% | 0.72 ± 0.09 | 3.34 ± 0.22 | 4.27 ± 0.35 | 4.70 ± 0.5 | 15.64 ± 0.95 |
| 1% | 0.38 ± 0.15 | 3.13 ± 0.24 | 4.49 ± 0.4 | 4.69 ± 0.48 | 16.20 ± 0.98 |

**Table 7.4** Mass specific oxygen consumption of embryos ( $\mu mol.g^{-1}h^{-1}$ ) of *Hemigrapsus edwardsii* in different developmental stages and in different salinities after 24 h at 15 °C. Values are mean ± S.E.M. of five or more replicates.

| Salinity of sea water (%) | $Mo_2$ ($\mu mol.g^{-1}h^{-1}$) | | | | |
|---|---|---|---|---|---|
| | Stage 1 | Stage 2 | Stage 3 | Stage 4 | Stage 5 |
| 100% | 1.52 ± 0.19 | 3.47 ± 0.23 | 4.48 ± 0.14 | 4.97 ± 0.32 | 18.07 ± 1.86 |
| 50% | 1.66 ± 0.26 | 3.42 ± 0.24 | 4.03 ± 0.41 | 4.99 ± 0.55 | 13.18 ± 1.86 |
| 10% | 1.31 ± 0.3 | 3.47 ± 0.22 | 4.28 ± 0.18 | 4.25 ± 0.79 | 9.47 ± 0.38 |
| 1% | 0.88 ± 0.26 | 3.05 ± 0.23 | 3.86 ± 0.36 | 4.68 ± 0.64 | 11.78 ± 1.35 |

**Table 7.5** Mass specific oxygen consumption of embryos ($\mu mol.g^{-1}h^{-1}$) of *Hemigrapsus edwardsii* in different developmental stages and in different salinities after 96 h at 15 °C. Values are mean ± S.E.M. of five or more replicates.

| Salinity of sea water (%) | $Mo_2$ ($\mu mol.g^{-1}h^{-1}$) | | | | |
|---|---|---|---|---|---|
| | Stage 1 | Stage 2 | Stage 3 | Stage 4 | Stage 5 |
| 100% | 1.59 ± 0.11 | 3.78 ± 0.26 | 2.46 ± 0.08 | 9.19 ± 0.89 | 8.21 ± 1.07 |
| 50% | 1.50 ± 0.11 | 3.73 ± 0.21 | 2.64 ± 0.21 | 8.80 ± 0.89 | 7.84 ± 0.55 |
| 10% | 2.97 ± 0.38 | 3.36 ± 0.19 | 2.82 ± 0.26 | 9.57 ± 0.52 | 9.22 ± 0.66 |
| 1% | 3.58 ± 0.29 | 2.95 ± 0.11 | 2.59 ± 0.48 | 8.48 ± 0.7 | 8.97 ± 0.34 |

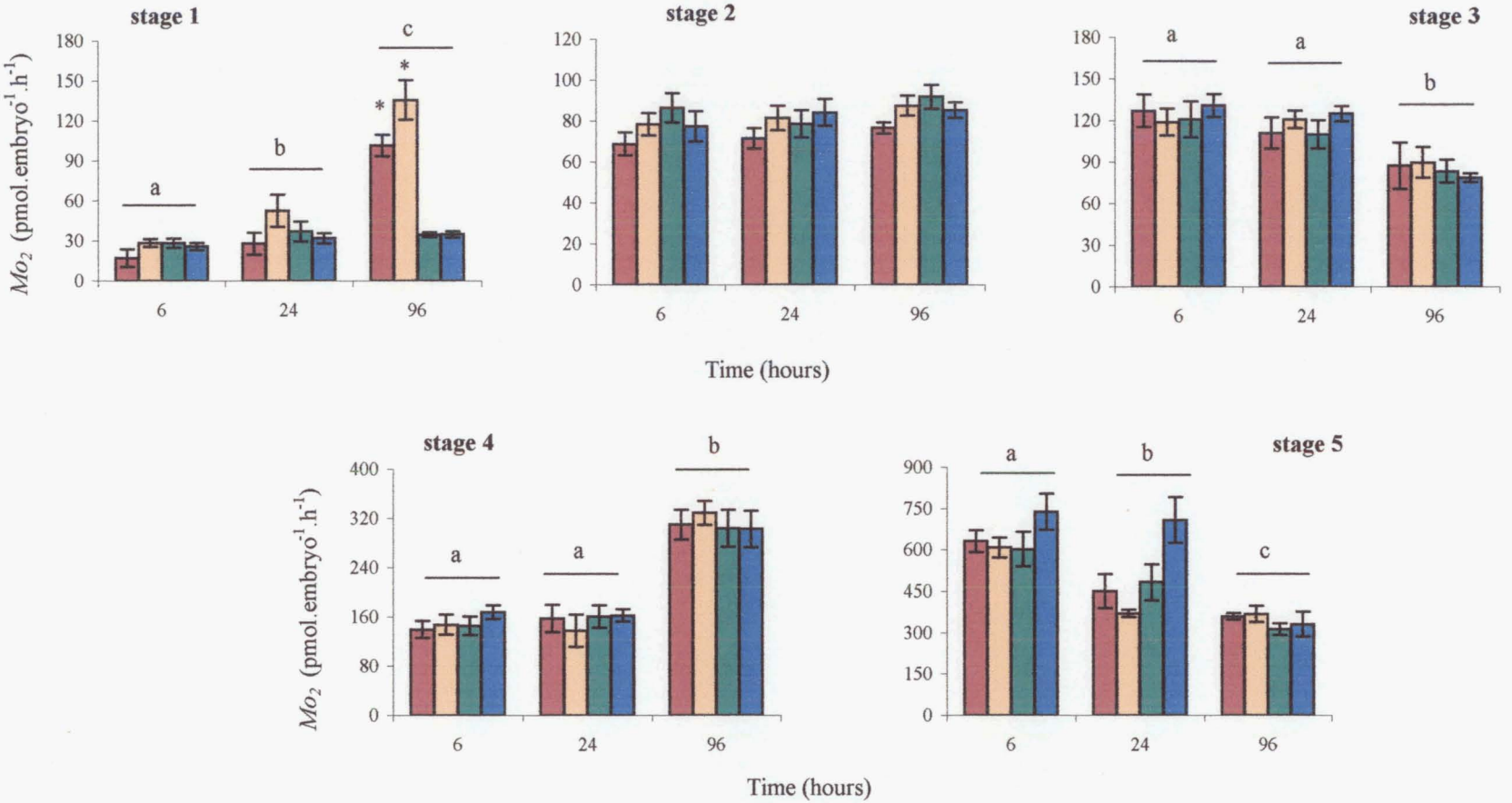



**Figure 7.3** Rates of oxygen consumption per embryo (pmol.embryo$^{-1}$.h$^{-1}$) of *Hemigrapsus edwardsii* at different developmental stages after exposure to four salinities for either 6, 24 or 96 h at 15 °C. ( 100%, 50%, 10% and 1% seawater). Each point is the mean ± S.E.M. of five or more replicates. Different letter labels (a, b and c) indicates significant differences among the group means at the three times at $P < 0.05$. Stage 1 embryos showed a significant interaction between salinity and exposure time. * After 96 h, oxygen consumption rates in 1% and 10% seawater were significantly higher than in 50% and 100% seawater at $P < 0.001$ (ANOVA Tukey post hoc).

in low salinities. However, a tendency for increased oxygen consumption in 50% seawater for all developmental stages was recorded for the time period of 6 to 24 h.

Figure 7.4 shows the oxygen consumption of a single embryo (pmol.embryo$^{-1}$.h$^{-1}$) of *H. crenulatus* at different salinities and times. As for *H. edwardsii*, stage 4 embryos showed an increase in the rate of respiration in different salinities with time. Two way ANOVA (salinity & time) on this stage, showed a significant effect of both salinity and time on the oxygen consumption (see ANOVA table below). The rate of oxygen consumption per embryo was decreased for stages 3 and 5 in dilute seawater with time and a significant effect of time was recorded (see ANOVA table below). There was an effect of the interaction factor salinity*time on the oxygen consumption of stage 2 embryos at $P < 0.02$ (ANOVA).

Results of two way ANOVA testing the effect of the factors salinity and time on the oxygen consumption on *H. crenulatus* embryos at each of 4 developmental stages are shown in the table below.

| | Salinity | | Time | | Salinity * Time | |
|---|---|---|---|---|---|---|
| | F | *P* | F | *P* | F | *P* |
| Stage 2 | $F_{(1),3,72} = 2.1$ | 0.104930 | $F_{(1),3,72} = 2.1$ | 0.000162 | $F_{(1),3,72} = 2.1$ | 0.022776 |
| Stage 3 | $F_{(1),3,54} = 2.0$ | 0.120302 | $F_{(1),3,54} = 6.5$ | 0.003061 | $F_{(1),3,54} = 0.5$ | 0.817772 |
| Stage 4 | $F_{(1),3,85} = 4.4$ | 0.006034 | $F_{(1),3,85} = 11.4$ | 0.000041 | $F_{(1),3,85} = 0.8$ | 0.511800 |
| Stage 5 | $F_{(1),3,52} = 0.3$ | 0.810515 | $F_{(1),3,52} = 120.5$ | 0.000000 | $F_{(1),3,52} = 0.9$ | 0.462708 |

Figure 7.4 indicates the results of the Tukey post hoc test.

Comparisons of the changes in $Mo_2$ of embryos (μmol.g$^{-1}$.h$^{-1}$) at stages 2 and 5 of both *H. edwardsii* and *H. crenulatus* with time are shown in Figures 7.5 and 7.6. Figure 7.5 shows the changes in the mass specific oxygen consumption in 50% seawater and Figure 7.6 shows that in 1% seawater. Interestingly, the pattern of rates of respiration was similar for both species. For both species, $Mo_2$ of stage 2 embryos was not affected with salinity and time. In contrast to that, $Mo_2$ of stage 5 embryos decreased significantly in 50% and 1% seawater ($P < 0.005$).

**Table 7.6** Mass specific oxygen consumption of embryos ($\mu mol.g^{-1}h^{-1}$) of *Hemigrapsus crenulatus* in different developmental stages and in different salinities after 6 h at 15 °C. Values are mean ± S.E.M. of five or more replicates.

| Salinity of sea water (%) | $Mo_2$ ($\mu mol.g^{-1}h^{-1}$) | | | |
|---|---|---|---|---|
| | Stage 2 | Stage 3 | Stage 4 | Stage 5 |
| 100% | 6.02 ± 0.42 | 9.61 ± 0.57 | 10.61 ± 0.43 | 18.89 ± 0.92 |
| 50% | 6.66 ± 0.42 | 10.02 ± 0.81 | 11.40 ± 0.53 | 20.69 ± 0.84 |
| 10% | 6.93 ± 0.6 | 9.14 ± 0.39 | 11.46 ± 0.62 | 20.00 ± 0.95 |
| 1% | 6.30 ± 0.35 | 8.31 ± 0.78 | 10.18 ± 0.85 | 19.68 ± 0.7 |

**Table 7.7** Mass specific oxygen consumption of embryos ($\mu mol.g^{-1}h^{-1}$) of *Hemigrapsus crenulatus* in different developmental stages and in different salinities after 24 h at 15 °C. Values are mean ± S.E.M. of five or more replicates.

| Salinity of sea water (%) | $Mo_2$ ($\mu mol.g^{-1}h^{-1}$) | | | |
|---|---|---|---|---|
| | Stage 2 | Stage 3 | Stage 4 | Stage 5 |
| 100% | 6.82 ± 0.39 | 8.82 ± 0.48 | 10.86 ± 0.41 | 13.04 ± 1.15 |
| 50% | 7.45 ± 0.63 | 9.78 ± 0.47 | 11.85 ± 0.65 | 14.73 ± 2.48 |
| 10% | 7.03 ± 0.31 | 9.62 ± 0.5 | 10.97 ± 0.91 | 14.57 ± 0.88 |
| 1% | 6.83 ± 0.37 | 9.03 ± 0.32 | 8.13 ± 0.83 | 14.72 ± 1.26 |

**Table 7.8** Mass specific oxygen consumption of embryos ($\mu mol.g^{-1}h^{-1}$) of *Hemigrapsus crenulatus* in different developmental stages and in different salinities after 96 h at 15 °C. Values are mean ± S.E.M. of five or more replicates.

| Salinity of sea water (%) | $Mo_2$ ($\mu mol.g^{-1}h^{-1}$) | | | |
|---|---|---|---|---|
| | Stage 2 | Stage 3 | Stage 4 | Stage 5 |
| 100% | 8.35 ± 0.19 | 9.57 ± 0.83 | 11.89 ± 0.89 | 8.28 ± 0.86 |
| 50% | 7.02 ± 0.73 | 9.33 ± 0.97 | 12.50 ± 0.59 | 6.90 ± 1.14 |
| 10% | 7.32 ± 0.44 | 7.74 ± 0.88 | 13.07 ± 1.08 | 6.17 ± 0.64 |
| 1% | 6.06 ± 0.46 | 7.10 ± 0.71 | 11.31 ± 1.06 | 4.04 ± 0.46 |

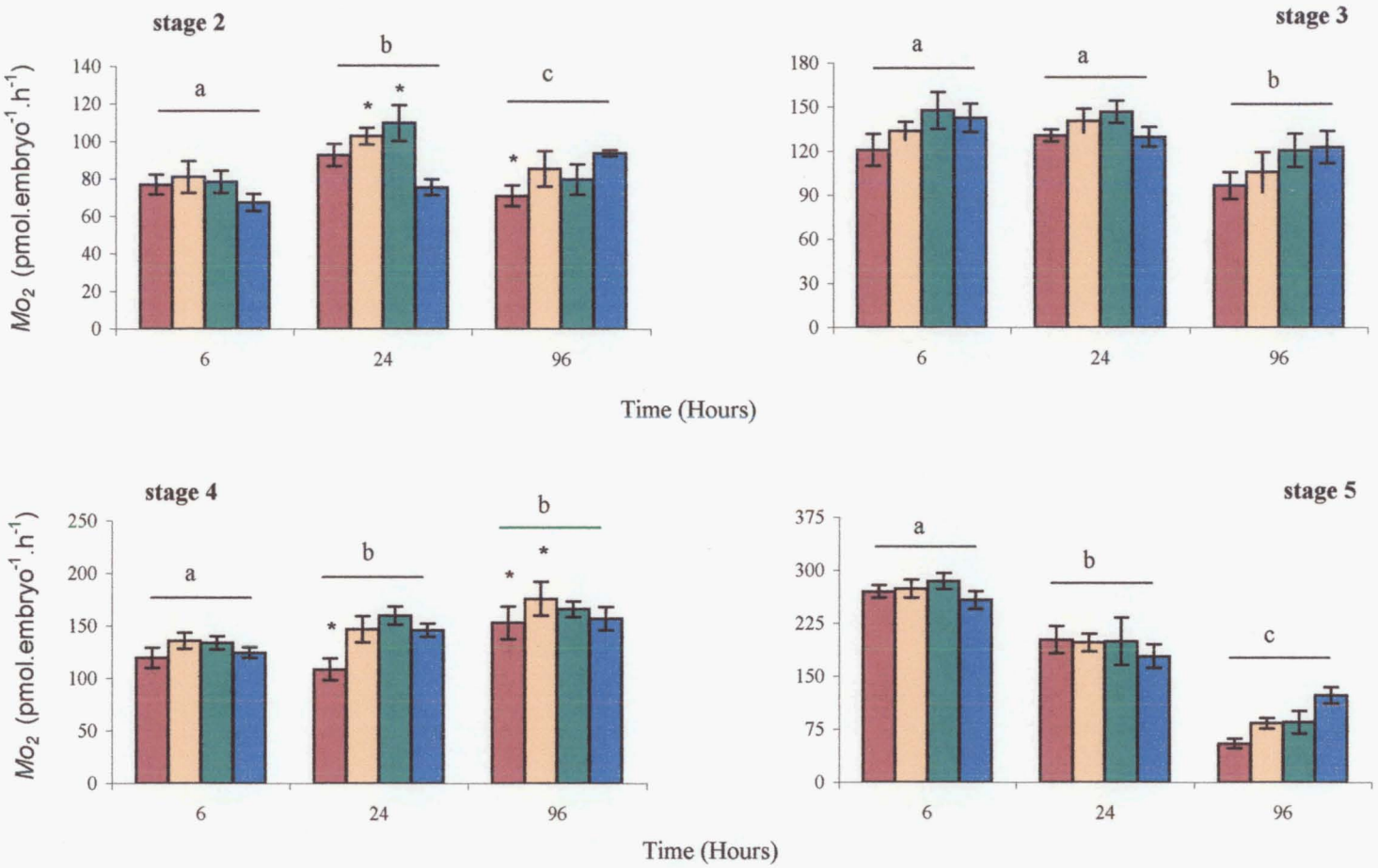



**Figure 7.4** Rates of oxygen consumption per embryo (pmol.embryo$^{-1}$.h$^{-1}$) of *Hemigrapsus crenulatus* at different developmental stages and salinities after either 6, 24 or 96 h at 15 °C in four salinities ( 100%, 50%, 10% and 1% seawater). Each point is the mean ± S.E.M. of five or more replicates. Different letter labels (a, b and c) indicate significant differences among the group mean at the three times at $P < 0.05$
* indicates means that are significantly diferent from the corresponding mean value for embryos in 100% sea water at each time at $P < 0.05$. (ANOVA Tukey post hoc).

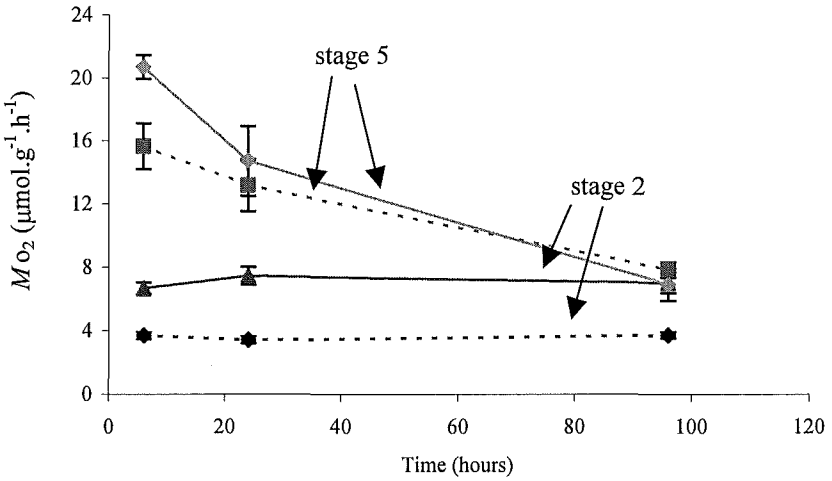



**Figure 7.5** Changes in the Mass specific oxygen consumption of stage 2 and stage 5 embryos of *Hemigrapsus edwardsii* ( ----- ) and *H. crenulatus* ( — ) in 50% seawater with time.

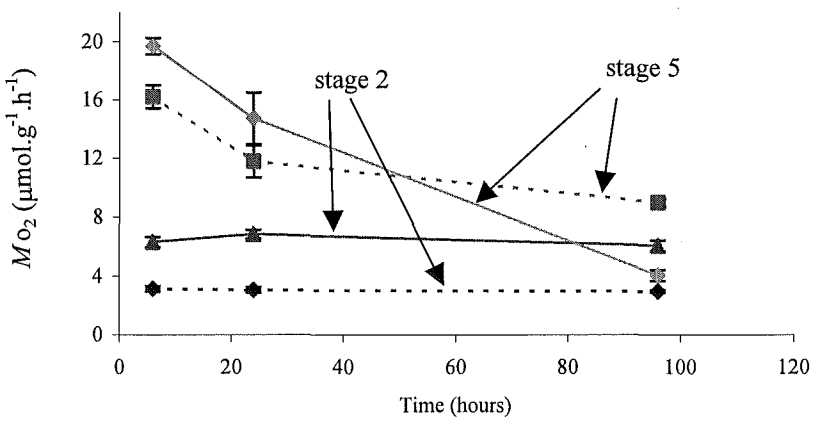



**Figure 7.6** Changes in the Mass specific oxygen consumption of stage 2 and stage 5 embryos of *Hemigrapsus edwardsii* ( ----- ) and *H. crenulatus* ( — ) in 1% seawater with time.

**Oxygen consumption of embryos of *H. edwardsii* continuously exposed to low salinities**

Figure 7.7 shows the time course of changes in the oxygen consumption per embryo (pmol.embryo$^{-1}$.h$^{-1}$) during development commencing either at stage 1 or stage 2 in 100%, 50% and 25% seawater. Development of embryos was delayed in dilute seawater as detailed in Chapter 3. Hatching of larvae in 100% seawater was observed after about 60 and 50 days in embryos commenced either at stage 1 and 2 respectively from the time of introduction to the experimental tanks. Development time of about 70 days was recorded for the embryos continuously exposed to 50% seawater in both experiments, i.e. for stage 1 or 2 embryos. In 25% seawater development of embryos did not proceed and abortion of embryos from the ovigerous crabs was noticed. Oxygen consumption increased with development in 100% and 50% seawater in embryos commenced at stage 1 and 2.

The time course of consumption of oxygen was generally similar in embryos incubated from stage 1 in 100% and 50% seawater. However, development was delayed in 50% seawater and oxygen consumption continued to increase after the 100% seawater embryos hatched (70 days). At day 20, oxygen consumption of the stage 1 embryos developed in 25% seawater was significantly elevated ($F_{(1),\ 2,\ 23}$ = 12.2, $P < 0.0002$, ANOVA, Tukey HSD) compared with that of embryos developed in 100% and 50% seawater.

Embryos commenced at stage 2 showed a significant difference in the oxygen consumption rates during development in 100% seawater and in dilution. When compared at the same times, the rate of respiration in the embryos developed in 50% seawater was lower than in 100% seawater, but it was reached to a similar or higher level at hatching (i.e. after 50 days in 100% seawater and after 60 days in 50% seawater) and subsequently exceeded the 100% value. In 25% seawater, this group also showed a peak in oxygen consumption at 20 days ($F_{(1),\ 2,\ 12}$ = 35.7, $P < 0.000009$) before declining and abortion after 50 days.

**Oxygen consumption of embryos of *H. crenulatus* continuously exposed to low salinities**

Figure 7.8 shows the oxygen consumption per embryo of *H. crenulatus* continuously exposed to three different salinities commencing either at stage 1 or at stage 2. As for *H. edwardsii*, development was delayed in dilute seawater. In 100% seawater successful hatching was occurred after about 37 and 31 days in the stage 1 and 2 embryos respectively whereas it took about 43 and 37 days in 50% seawater.

Rates of respiration of stage 1 embryos were similar during development in 100% and 50% seawater. The rates of uptake of oxygen in corresponding stages were relatively similar in 100% and 50% seawater. Significant differences in the oxygen consumption of a single egg of stage 1 have been recorded after 3 days in dilute seawater with that of 100% seawater ($F_{(1),\ 2,\ 6} = 8.6$, $P < 0.017$). Stage 1 embryos developed in 50% seawater shown an increase in the oxygen consumption rate when they were close to hatch. Oxygen consumption rates after 43 days for the embryos developed in 50% seawater were comparable to the oxygen consumption rates of embryos developed in 100% seawater after 37 days for stage 1 embryos.

In contrast to *H. edwardsii*, stage 2 embryos developed in 50% seawater demonstrated higher oxygen consumption rates at each sample time compared to those in 100% seawater. However, taking into account delayed development in 50% seawater, the rates of up take of oxygen at corresponding stages were relatively similar in 100% and 50% seawater (i.e. a comparable rates of oxygen consumption were recorded after 31 days and 37 days for the embryos developed in 100% seawater and 50% seawater). In 25 % seawater, oxygen consumption rates didn't change markedly and it was very low compared to 100% seawater. Significant differences in the oxygen consumption rates in the three different salinities (100%, 50% and 25% seawater) have been recorded during the later development times after 25 days ($F_{(1),\ 2,\ 6} = 34.3$, $P < 0.00521$).

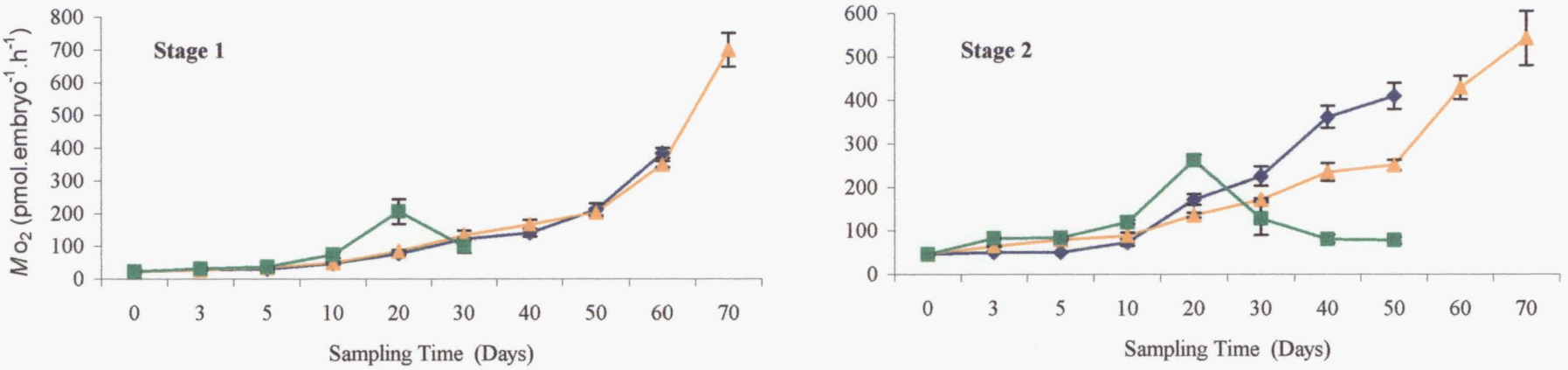



**Figure 7.7** Oxygen consumption per embryo (pmol.embryo$^{-1}$.h$^{-1}$ ) of *Hemigrapsus edwardsii* developed in different salinities from stage 1 & 2 at 15 °C. (—◆— 100%, —▲— 50% and —■— 25% seawater). Values are mean ± S.E.M. of 5 - 10 more replicates. Statistical significances are indicated in the text.

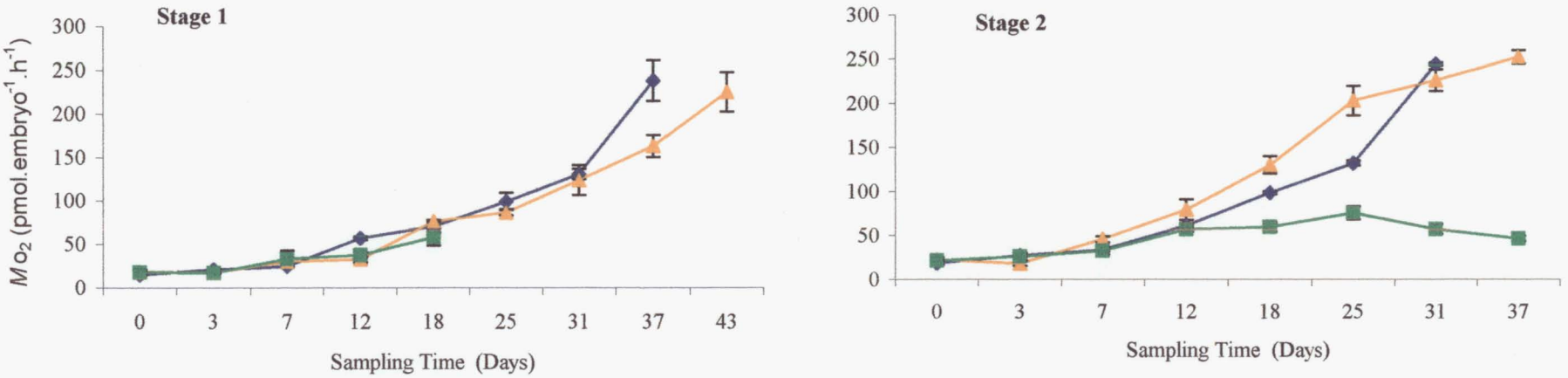



**Figure 7.8** Oxygen consumption per embryo (pmol.embryo$^{-1}$.h$^{-1}$ ) of *Hemigrapsus crenulatus* developed in different salinities from stage 1 & 2 at 15 °C. (—◆— 100%, —▲— 50% and —■— 25% seawater). Values are mean ± S.E.M. of 3 - 8 more replicates. Statistical significances are indicated in the text.

**Total cost of development of a single embryo of *H. edwardsii* and *H. crenulatus***

Table 7.9 shows the calculated mean values (± S.E.M.) of the total cost of development of a single embryo of *H. edwardsii* and *H. crenulatus* in 100% and 50% seawater commencing either at stage 1 or 2 at 15 °C. It can be seen that in 100% seawater, the total cost of development of a single embryo of *H. crenulatus* is about 2.5 times and 3.5 times less than that of *H. edwardsii* commencing at either stage 1 or 2 respectively. There were significant differences in the total cost of development from extrusion (stage 1) to hatching or from gastrula stage (stage 2) to hatching in 50% seawater compared to 100% seawater for both species (Significant levels are given in the table). Note that as the development time was longer in 50% seawater, this contributed to the total cost of development in 50%.

**Table 7.9** Total cost of development of a single embryo of *H. edwardsii* and *H. crenulatus* in 100% and 50% seawater commencing either at stage 1 or at 2 at 15 °C. Values are mean ± SEM. (n = 5). (*, ** statistically significant @ $P < 0.05$ and $P < 0.02$ respectively, between 100% and 50% seawater within each species and stage).

| | Total cost of development of a single embryo (nmol $O_2$) | | | |
|---|---|---|---|---|
| | From **Stage 1** | | From **Stage 2** | |
| | 100% seawater | 50% seawater | 100% seawater | 50% seawater |
| *H. edwardsii* | 210.58 ± 7.36** | 323.72 ± 19.75** | 303.27 ± 23.21* | 386.68 ± 25.77* |
| *H. crenulatus* | 85.39 ± 6.59** | 118.87 ± 10.58** | 84.16 ± 3.86* | 136.68 ± 9.13* |

## DISCUSSION

For both species, blastula stage embryos had the lowest respiration rates per unit mass and per embryo. Significant increase in the oxygen consumption of a single embryo during development was observed. Similar pattern of increase in the rates of respiration in developing embryos of *Heterozius rotundifrons* and *Cyclograpsus lavauxi* has been reported by Taylor & Leelapiyanart (2001). This presumably reflects the transformation of non-respiring storage material into respiring embryonic tissue as the embryo develops, and the respiration rate of the whole embryo naturally increases (Quetin & Ross, 1989).

The increase in oxygen consumption of single embryos during development was steepest towards the end of the incubation period (Figure 7.1 & 7.2). This may be explained as an increase demand for energy attributable to movements within the embryo such as larval heartbeat or muscular activity of the whole, encapsulated larva and by the greater mass of respiring tissue. Also, this observation is correlated with the observed increase in $Na^+/K^+$ ATPase activity in these embryos which is known to be a major component of total metabolism (Chapter 5). The usage of different substrates of metabolism at different embryonic stages may also contribute to differences in $Mo_2$ with development. There is some evidence that this is the case in crab embryos (Babu, 1987; Pandian, 1970). Naylor *et al.*, (1999) have documented that the rates of oxygen uptake of early stage embryos may be related to proportional changes in size and oxygen demand of the embryos, which in later stages may be enhanced by the development of embryonic haemocyanin.

Rates of oxygen consumption of embryos in acute exposure to dilute seawater were variably increased or decreased for each developmental stage and also with time for both species (Figure 7.3 & 7.4). It is unclear whether the increase in the metabolic rates of stage 1 embryos of *H. edwardsii* is due to any physiological activity as viability of those embryos in dilute seawater is low. Interestingly, the rate of respiration for embryos at stages 3 & 5 embryos of both species decreased with time whereas it was increased for stage 4 embryos in all salinities. Therefore, these changes seem to reflect time isolated in media rather than salinity.

The long term experiment showed, except for stage 2 embryos of *H. crenulatus*, that the oxygen consumption rates of single embryos continuously exposed to 50% seawater were generally comparable with those of corresponding embryo stages developed in 100% seawater (Figures 7.7 & 7.8). However, a slower rate of development leads to higher total cost of development in 50% seawater. An increase was observed in the rate of yolk depletion of embryos developed in 50% seawater (Chapter 3, Figure 3.9). However, this aspect needs to be further investigated with quantitative measurements of yolk utilization during development and in dilution. These observations lead to the question whether the hatching larvae in 50% seawater with relative small yolk quantity can develop successfully until they start feeding. This was not investigated here.

Oxygen consumption rates of stage 2 embryos of *H. crenulatus* were significantly increased when continuously exposed to 50% seawater (Figure 7.8). Interestingly, this observation can correlate with the results obtained in the study of the changes in $Na^+$ / $K^+$ ATPase activity of the embryos of *H. crenulatus* continuously exposed to 50% seawater from stage 2 to hatching (Chapter 5). $Na^+/K^+$ ATPase activity of the embryos developed in 50% seawater was significantly increased by Yolk in 4 lobe stage. It is known that active pumping of ions via $Na^+$ / $K^+$ ATPase is one of the physiological mechanisms that crustaceans utilize to control osmolarity, which is energetically costly (Alberts *et al.*, 1989; Dall via, 1970; Magnum & Towle 1977; Welcomme & Devos, 1988). Therefore, a correlation of increased oxygen consumption and ATPase activity is possible for the stage 2 embryos developed in 50% seawater.

The metabolic cost of development (from stage 1 to hatching), in terms of oxygen consumption, of single embryos of *H. edwardsii* and *H. crenulatus* were 210.58 ± 7.36 and 85.39 ± 6.59 nmol $O_2$ respectively. Calculated cost of development of a single embryo in 50% seawater either commencing from stage 1 or stage 2 to hatching for both species was higher than that of 100% seawater (Table 7.9). Prolonged development and delayed hatching of embryos in 50% seawater contributed to this increased calculated value as no increase in the oxygen uptake of corresponding stages were observed in 50% seawater with that of 100% seawater.

Passive mechanisms such as changes in membrane permeability (Lucu, 1990; Potts & Parry, 1963; Shaw, 1955; Sutcliffe, 1961) and cellular release of osmotic effectors (Gills, 1977; Pierce, 1982) could also be employed by these embryos to maintain internal osmolality requiring less energy. This phenomena needs to be investigated with these embryos as changes in the permeability of embryo membranes was not examined in this present study.

In conclusion, the oxygen consumption rates of embryos of both species *H. edwardsii* and *H. crenulatus* showed no related effect of reduced salinity in the acute experiments. This suggests, that within the tolerable range of salinity oxygen consumption remain essentially unaffected. Differences in oxygen consumption rates in the acute experiments were probably artefacts related to the time isolated in media rather than to salinity. Utilization of yolk reserves as an energy source may probably contribute to have relatively unaffected oxygen consumption rates by these embryos when continuously exposed to dilute seawater as no salinity related increase in metabolic rates observed. The significant increase in the calculated total cost of development in 50% seawater is due to the delayed development rather than the osmotic work requires to maintain salt and water balance.

# CHAPTER EIGHT

## CONCLUSIONS

In marine species, salinity and its potential variations are among the key factors with which the organisms have to cope. As the development of embryos of two intertidal crabs, *Hemigrapsus edwardsii* and *Hemigrapsus crenulatus* is external, attached to the abdomen of the maternal crab for several months, the embryos must be exposed to variations of salinity in the environment. Thus, the aim of this thesis was to investigate the effects of variations in salinity on the development of embryos of the two crab species in relation to acute and continuous exposure from spawning to hatching.

The ontogeny of osmoregulation has now been documented from the early stages of embryonic development in both species. It was hypothesised that ability to survive and mechanisms of osmoregulation in these two species varied due to the variations in the habitats of the two crab species. However, there were only minor differences in the survival and osmoregulatory abilities found between the two species. In both species, the capacity for osmoregulation is correlated with the appearance of putative osmoregulatory epithelium. This observation was further investigated with the embryos of *H. crenulatus* and it was supported with increased $Na^+/K^+$ ATPase activity and efficient ion regulation during development. Short turnover rates of water and sodium in the embryos of *H. crenulatus* confirmed active osmoregulation in these embryos.

Both *H. edwardsii* and *H. crenulatus* showed a similar pattern of embryonic development as for other crabs (Bas, 2000; Helluy & Beltz, 1991; Leelapiyanart, 1996; Subramonian, 1979). Embryos of *H. edwardsii* are bigger and had a relatively longer period of incubation compared to embryos of *H. crenulatus*. This time variation may possibly due to the size of the embryo as increasing embryo size slows down the rate of development (Wear, 1974).

Salinity tolerance experiments revealed that the post gastrula stage embryos of both species are very tolerant to dilute seawater. Mortality rates were high for stage 1 embryos in hyposaline waters and increased with exposure time for both species (Figure 3.1 and 3.2). Compared to *H. edwardsii*, stage 5 embryos of *H. crenulatus* has shown low survival rates in dilute seawater. Similarly, Leelapiyanart (1996) reported the ability to tolerate low salinities in the late embryonic developmental stages of *Heterozius rotundifrons* and *Cyclograpsus lavauxi*. In both species, normal development and successful hatching from gastrulation could occur in 50% seawater. In contrast, pregastrula stage embryos (Stage 1) showed low survival rates in dilute seawater and were unable to complete development and hatch normally in 50% seawater (Chapter 3). A plausible explanation for high survival rates at late stages of embryonic development in these two species may be the capability to osmoregulate. During ontogeny, a strong correlation has often been observed in several crustacean species, between an increasing ability to osmoregulate and an improving salinity tolerance (Charmantier & Charmantier-Daures, 1991, 1994; Charmantier *et al.*, 1998; Leelapiyanart, 1996; Morritt & Spicer, 1995). Future studies on the behavioural adaptations of female crabs carrying newly spawned embryos may support the hypothesis that they avoid harsh environmental conditions until the embryos develop the mechanism to osmoregulate.

Osmolality experiments found that the embryos of both species, except stage 1, were hyperosmotic to the medium throughout development. Stage 1 embryos of both species osmoconformed in all salinities during the observation times. The osmolality of crab embryos decreased with the decrease in salinity. In both species, there was a general fall in osmolality of embryos after 96 h exposure time with decreasing salinity. In contrast to *H. edwardsii*, stage 5 embryos of *H. crenulatus* tended to move to isosmotic condition after 96 h in dilute seawater.

In the long term exposure experiment, it was found that the total embryo osmolality decreased rapidly during the first few days of introduction of pregastrula embryos (stage 1) of both species to the low salinities, whereas the rate of fall of osmolality of post gastrula embryos in low salinities was slower. It appears that the capacity to osmoregulate commences at the gastrula stage for these embryos. Moreover, it can be said that gastrulation marks the critical stage for these embryos where a transition

from isosmotic to hyper-osmotic regulation occurs. Therefore, the osmotic shock which these early developing embryos (stage 1) face during these important morphogenetic changes may be the reason for the failure of the stage 1 embryos to develop normally in dilute seawater. These observations are clearly associated with the occurrence of osmoregulatory mechanisms by the gastrula stage.

Ion regulation as an integral part of the osmotic and volume regulation of embryos of different species has been investigated by several authors (Brown & Terwilliger, 1992; Leelapiyanart, 1996, Potts & Ruddy, 1969). Therefore, contribution of inorganic ions in the osmoregulation in these embryos was investigated during embryonic development and in relation to salinity stress conditions. This set of experiments were confined to the embryos of *H. crenulatus* only. It was assumed that the osmotically active inorganic ions are distributed in the solvent volume of these eggs. Solvent volume is the free water in the embryo (Chapter 3). The four cations $Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$ (and associated anions) were major osmotic effectors in the embryos of *H. crenulatus* but contributed only about half of the total osmolality in later stage embryos. Therefore, other osmotically active materials such as free amino acids, yolk products and other organic metabolites must play an important part in volume regulation in these embryos. Although the overall concentrations of the four cations decreased as external salinity decreased in all embryo stages, they were quite well regulated in postgastrula stage embryos. The change in internal osmolality (due to sodium, potassium, calcium and magnesium) with dilution for early stage embryos is associated primarily with ion loss. However, for later stages, the change in internal osmolality on dilution is associated with loss of other osmolytes.

Mechanisms responsible for osmoregulatory abilities is linked to high levels of $Na^+/K^+$ ATPase activity (Charmantier *et al.* 2001; Flik & Haond, 2000; Harris, 1993; holliday *et al.*, 1990; Lee & Watts, 1994). Thus the demonstrated abilities of the embryos of *H. crenulatus* to survive in dilute seawater and to hyperosmoregulate throughout development led to hypothesise the possession of certain physiological mechanisms in osmoregulationin these eggs. Therefore, evidence for involvement of $Na^+/K^+$ ATPase in osmoregulation in these embryos was studied on a short term and long term basis in response to hyposaline exposure. The enzyme activity was not detectable for stage 1 embryos. It was found that there is a significant increase in the

ATPase activity during development commencing from the gastrula stage (stage 2). The increase in enzyme activity during early development of the embryos of *H. crenulatus* could possibly be related to the synthesis of the enzyme necessary for a successful transition to an osmoregulatory mechanism. There was an increase in the enzyme activity in all postgastrula stage embryos when acutely exposed to dilute seawater. The enzyme activity was significantly increased in the embryos that were incubated by crabs and continuously exposed to 50% seawater was observed from the gastrula stage. Short term responses to sudden salinity changes may be based on the activation of the existing enzymes whereas longer term acclimation may be based on the synthesis of new $Na^+/K^+$ ATPase molecules. It appears that the postgastrula stage embryos of *H. crenulatus* have a potential for elevating $Na^+/K^+$ ATPase activity, and are therefore capable of acclimating to hyposaline environments.

Mechanisms underlying the osmotic protection of aquatic embryos are currently controversial, for crustaceans as well as for other marine groups (Charmantier *et al.*, 2001). In the present study, it was investigated whether the eggs of *H. crenulatus* develop as a closed system with an impermeable barrier to the passage of water or ions across the surface or exist in a dynamic steady state performing osmotic work involving active uptake of ions and active excretion of water. This was examined by using fluxes of traces of $^{22}Na$ and $^3H_2O$ for all developmental stages of *H. crenulatus* in 100% seawater. Turnover times for water and sodium were considerably shorter than the acclimation period during which hyperosmoregulation was demonstrated, indicated that the embryo membranes are effectively permeable to water and ions throughout development. Therefore, the current hypothesis (Charmantier *et al.*, 2001) that decapodan embryos possess highly impermeable embryo membranes is not applicable to these embryos. There was a decrease in the diffusive water permeability but an increase in sodium turnover with development of embryos. It is found that exchangeable water and sodium comprised of two compartments, i.e. a rapidly exchanging pool and a slowly exchanging pool. Morphological studies revealed that these embryos possess two distinct membranes surrounding the embryo (inner and outer membranes) which may act as potential permeability barriers. On the basis of these observations, it is likely that the rapidly exchanging pool is the perivitelline space in these embryos and the embryo/yolk represents the inner pool.

Total exchangeable sodium increased with development. This is consistent with the AAS analysis and similar net uptake of sodium has been shown in other studies (Pandian, 1970). However, the most important conclusion that can be made from my study is that these embryos are in a dynamic steady state with respect to sodium and water. As they are hyperosmotic (Chapter 3), they must be actively osmoregulating and either the influx or efflux of $Na^+$ should be performed by active transport.

Since osmoregulaton requires metabolic energy, oxygen consumption rates as a measure of metabolic rates were determined during development and in hyposaline exposure of embryos of *H. edwardsii* and *H. crenulatus* at 15 °C. It was hypothesised that the metabolic rates of the developing embryos could affect with changing salinity as it is generally accepted that organisms experiencing physiological stress expend more energy in coping with that stress (Richmond & Woodlin, 1999). Stage 1 embryos (cleavage to blastula) of both species showed the lowest oxygen consumption per unit mass and per embryo. A progressive increase in the oxygen uptake of embryos with development was recorded for both species. Rates of oxygen consumption of embryos in acute exposure to dilute seawater varied for each developmental stage and also over time for both species. These changes in oxygen consumption rather seem to reflect time isolated in media rather than salinity and therefore were probably artifacts.

The calculated metabolic cost of development in 50% seawater was higher for both species compared to that in 100% seawater. Development of embryos in 50% seawater was delayed. However, there was no significant increase in the oxygen consumption rates in comparable stages in the embryos developed in 100% and 50% was observed. Therefore, prolonged development and delayed hatching of embryos in 50% seawater was the main reason for this increased metabolic cost of development. This is a real cost and potentially very important for success of embryo. The fact that utilization of yolk as an energy source in dilute seawater needs further investigation requiring quantitative measurements of yolk depletion and determination of the pattern of yolk utilization. Another possible mechanism to minimise the cost of osmoregulation in dilute seawater is by reducing permeability of the body surface to water and to salts. (Potts & Parry, 1963; Shaw, 1955; Sutcliffe, 1961). This was not

examined in the present study, but I suggest future studies should investigate permeability changes in egg membranes in dilute seawater.

It has been hypothesied that the ontogeny of osmoregulation is correlated with the differentiation of ion-transporting tissues (Bouaricha *et al.*, 1991; Charmantier, 1998; Felder, 1986; Lee & Watts, 1994). It is showed that the developing eggs of both *H. edwardsii* and *H. crenultus* have the ability to osmoregulate before the appearance of any obvious organ responsible for osmoregulation as in adults. Several authors documented the presence of a transitory organ in crustacean embryos which function as an embryonic osmoregulatory organ during embryonic development (Conte, 1972; Ewing *et al.*, 1974; Hootman *et al.*, 1972; Hootman & Conte, 1975; Morritt & Spicer, 1995). Localization of ion permeable area/areas in the embryos of *H. edwardsii* and *H. crenulatus*, using the silver nitrate staining method revealed a dark-staining patch associated with the embryo membranes from gastrula stage in both species. In stage 1 embryos, silver staining was more diffuse and patchy. In all postgastrula stages, the staining patch was located adjacent to the yolk, not the embryo. These observations suggest that the stained area is a site where chloride ions are diffusing out of the embryo. Composition of the deposition investigated with dispersive x-ray microanalysis confirmed as silver and chloride as major constituents. The appearance of this "osmoregulatory epithelium" from gastrula stage supports the findings that the capacity to osmoregulate commences at gastrula stage in both species.

Osmoregulatory studies on adult crustaceans have generally referred to measurements on haemolymph samples. However, the small size of the eggs of both *H. edwardsii* and *H. crenulatus* prevented sampling from specific intercellular or extracellular fluids and homogenized egg samples were used. Osmolality measurements were completed within 1-2 min after homogenization. A possible source of error in osmolality measurements on cell homogenates is the release of osmotically active solutes during cellular lysis. This was investigated by doing time series of measurements of the homogenates for all developmental stages. No marked change in the osmolality of the homogenate was noticed even after 10 minutes.

The conclusions of osmoregulation or osmoconforming by crab embryos are not invalidated by the use of homogenates of whole embryos that presumably contained

intracellular, extracellular and extraembryonic fluids. Since intracellular/extracellular fluids in animals are generally close to isosmotic, this probably had little effect on the osmoregulation curves in these embryos. Furthermore, inclusion of any extraembryonic fluids in equilibrium with the external medium would have made the embryos appear to be weaker regulators than they really are. In Chapter 6, it was shown that these embryos are consisted of two clear pools, "fast" and "slow". It was concluded that the fast pool represents the perivitelline space in these eggs and the slow pool represents the embryo. The size of the slow pool increased with development whereas it was not changed in the fast pool.

With respect to ionic regulation the situation is more complex because intracellular, extracellular and external media generally have quite different ionic composition. So that the actual concentrations and the regulation curves may be distorted by "contamination". Several studies have shown that during changing salinity cells tend to protect intracellular ion concentrations and instead adjust the free amino acid pool and other solutes like taurine, betaine, TMAO (Yancy *et al.*, 1982). There are two possible advantages of this. (1). To preserve ion concentrations concerned with electrical activity/excitability (ions are perturbing solutes). (2). To preserve protein structure and enzyme activity by adjusting the compatible solutes (Yancy *et al.*, 1982). Clearly (1) and (2) could be important for maintaining normal metabolism and development in the embryos. In the present study, the data suggest that a similar strategy of adjusting compatible osmolytes may be occurring in crab embryos, although further studies would be needed to determine the involvement of these osmolytes in osmoregulation, and their mechanisms. Such mechanisms have been reported in adult *H. edwardsii*. An increase in the intracellular amino acid levels in hyperosmotic exposure (Leader & Bedford, 1978) was described as an adaptive feature of osmotic readjustment in these crabs.

Finally, my results refute the current premise that decapodan embryos are osmoconformers and possess impermeable egg membranes (Charmantier & Charmantier-Daures, 2001) at least for these two *Hemigrapsus* species which maintain a hyperosmotic state throughout development and have highly permeable embryo membranes. Likewise, the capacity to osmoregulate occurs at much earlier

stages in these embryos than the stages suggested by Bouaricha *et al.* (1994) and Felder *et al.* (1986).

Hyperosmoregulation must involve a site of ion uptake. From my data it is apparent that the embryos of both species must gain water continuously by osmosis. Therefore, these embryos should possess a site for active uptake of ions and excretion of salt and water. It is possible that epithelial structure associated with the silver-staining patch may function as the embryonic osmoregulatory organs in these embryos. Figure 8.1 shows a suggested model for ion uptake and salt and water excretion in these embryos.

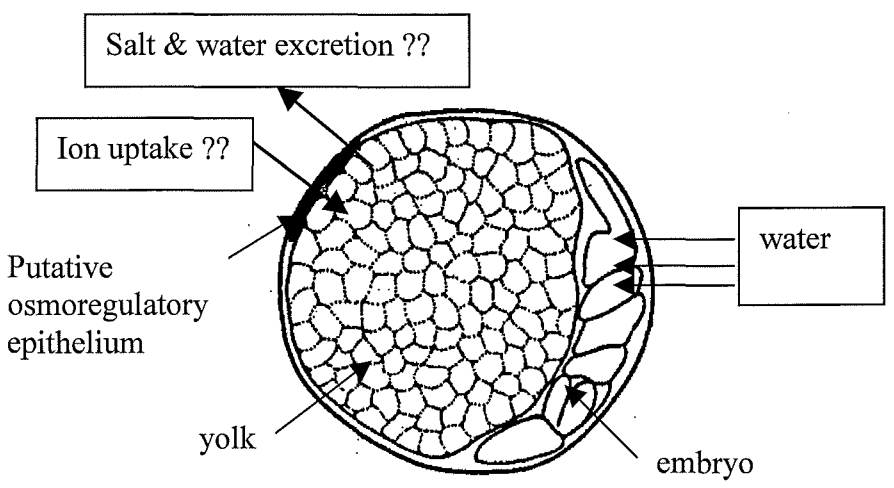



**Figure 8.1** Suggested model for ion uptake and salt and water excretion in the two *Hemigrapsus* embryos.

I suggest that this aspect could be further investigated with immunocytochemical studies in future localizing biological molecules involved in active transport system in these eggs. Also, looking at gene expression of a variety of transporters in this tissue may worth studying.

# REFERENCES


**Aarset, A. V. and Aunaas, T. (1990).** Effects of osmotic stress on oxygen consumption and ammonia excretion of the Arctic sympagic amphipod *Gammarus wilkitzkii. Mar. Ecol. Prog. Ser.* 58: 217-224.

**Aarset, A. V. and Aunaas, T. (1990).** Influence of environmental salinity on oxygen consumption and ammonia excretion of the arctic under-ice amphipod *Onisimus glacialis. Mar. Biol.* 107: 9-15.

**Ahearn, G. A., Duerr, J.M., Zhuang, Z., Brown, R.J., Aslamkhan, A. and Killebrew, D.A. (1999).** Ion transport processes of crustacean epithelial cells. *Physiol. Biochem Zool.* 72(1): 1-18.

**Aladin, N. V. and Potts, W.T.W. (1995).** Osmoregulatory capacity of the Cladocera. *J. Comp. Physiol B.* 164: 671-683.

**Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. (1989).** *Molecular biology of the cell*, 2nd edition, Garland Publishing, Inc., New York.

**Aldridge, J. B. and Cameron, J.N. (1982).** Gill morphometry in the Blue Crab, *Callinectes sapidus* Rathbun (Decapoda, Brachyura). *Crustaceana.* 43(3): 297-305.

**Almatar, S. M. (1984).** Effects of acute changes in temperature and salinity on the oxygen uptake of larvae of herring (*Clupea harengus*) and plaice (*Pleuronectes platessa*). *Mar. Biol.* 80: 117-124.

**Amende, L. and Pierce S.K. (1980).** Free amino acid mediated volume regulation of isolated *Noetia podorosa* red blood rolls: control by $Ca^{2+}$ and ATP. *J. Crust. Physiol.* 138: 291-298.

**Amsler, M. O. and George R.Y. (1984).** Seasonal variation in the biochemical composition of the embryos of *Callinectes sapidus* Rathbun. *J. Crust. Biol.* 4: 546-553.

**Anderson, D. T. (1973).** *Embryology and Physiology in Annelids and Arthropods.* Oxford, Pergamon Press Ltd. Pp 495.

**Anderson, O. S. (1978).** Permeability properties of unmodified lipid bilayer membranes. In Giebisch, G., Tosteson, D.C., Ussing, H.H., eds., *Membrane Transport in Biology*: New York, Springer-Verlag. 1: 508-509.

**Anger, K. and Schultze, K. (1995).** Elemental composition (CHN), growth and exuvial loss in the larval stages of two semiterrestrial crabs, *Sesarma curacaoense* and *Armases miersii* (Decapoda : Grapsidae). *Comp. Biochem. Physiol.* 111A (4): 615-623.

**Anger, K. (1996).** Salinity tolerance of the larvae and first juveniles of a semiterrestrial grapsid crab, *Armases miersii* (Rathbun). *J. Exp. Mar. Biol. Ecol.* 202: 205-223.

**Anger, K., Riesebeck , K. and Puschel, C. (2000).** Effects of salinity on larval and early juvenile growth of an extremely euryhaline crab species, *Armases miersii* (Decapoda: Grapsidae). *Hydrobiologia.* 426: 161-168.

**Anger, K. and Charmantier, G. (2000).** Ontogeny of osmoregulation and salinity tolerance in a mangrove crab, *Sesarma curacaoense* (Decapoda: Grapsidae). *J. Exp. Mar. Biol. Ecol.* 251: 265-274.

**Attard, J. and Hudon, C. (1987).** Embryonic development and energetic investment in egg production in relation to size of female lobster (*Homarus americanus*). *Can. J. Fish Aquat. Sci.* 44: 1157-1164.

**Avenet, P. and Lignon J.M. (1985).** Ionic permeabilities of the gill lamina cuticle of the crayfish, *Astacus leptodactylus* (E). *J. Physiol.* 363: 377-401.

**Babu, D. E. (1987).** Observations on the embryonic development and energy source in the crab *Xantho bidentatus. Mar. Biol.* 95: 123-127.

**Baeza, J. A. and Fernandez, M. (2002).** Active brood care in *Cancer setosus* (Crustacea: Decapoda): the relationship between female behaviour, embryo oxygen consumption and the cost of brooding. *Functional Ecology.* 16: 241-251.

**Barnes, H. (1954).** Some tables for the ionic composition of sea water. *J. Exp. Biol.* 31 (4) 582-588.

**Barra, J., Pequeux, A. and Humbert, W. (1983).** A morphological study on gills of a crab acclimated to fresh water. *Tissue & Cell.* 15(4): 583-596.

**Bas, C. C. and Spivak, E.D. (2000).** Effect of salinity on embryos of two southwestern Atlantic estuarine grapsid crab species cultured *in vitro. J. Crust. Biol.* 20(4): 647-656.

**Beadle, L. C. (1969).** Salt and water regulation in the embryos of freshwater pulmonate molluscs, III. regulation of water during the development of *Biomphalaria sudanica. J. Exp. Biol.* 50: 491-499.

**Bedford, J. J. and Leader, J.P. (1977).** The composition of the haemolymph and muscle tissue of the shore crab, *Hemigrapsus edwardsi,* exposed to different salinities. *Comp. Biochem. Physiol.* 57A: 341-345.

**Bedford, J. J. and Leader, J.P. (1978).** Hyperosmotic readjustment of the crab, *Hemigrapsus edwardsi. J. Comp. Physiol* 128: 147-151.

**Begg, S. M. C. (1980).** Factors affecting the distribution of two species of intertidal grapsid crabs. B.Sc. (Hons) project. Dept. of Zoology, University of Otago. 82 pp.

**Bennet, E.W. (1964).** The marine fauna of New Zealand: Crustacea, Brachyura. *N.Z. Dept. Sci. Industr. Res. Bull.* 153 (N.Z. Oceanogra. Inst. Mem. 22), 120p. (cited in McLay, 1988).

**Bloomfield, G. (1982).** Effects of season and salinity acclimations on survival, permeability and osmoregulation of *Hemigrapsus crenulatus* (Crustacea: *Grapsidae*). M.Sc. Thesis, University of Canterbury, 118p.

**Boeck, G. D., Vlaeminck, A., Linden, A.V. and Blust, R. (2000).** The energy metabolism of Common Carp (*Cyprinus carpio*) when exposed to salt stress: An increase in energy expenditure or effects of starvation? *Physiol. Biochem. Zool.* 73(1): 102-111.

**Boolltian, R. A., Giese, A. C., Farmanfarmain & Tucker, J. (1959).** Reproductive cycles of five west coast crabs. *Physiol. Zool.* 32: 213-220.

**Boone, E. and Bass-Becking, G.M. (1931).** Salt effects on eggs and nauplii of *Artemia salina* L. *J. Gen. Physiol.* 14: 753-763.

**Bosque, T., Hernandez, R., Perez, R., Todoli, R. and Oltra, R. (2001).** Effects of salinity, temperature and food level on the demographic characteristics of the seawater rotifer *Synchaeta littorialis* Rousselet. *J. Exp. Mar. Biol. Ecol.* 258: 55-64.

**Bouaricha, N., Thuet, P., Charmantier, G., Charmantier-Daures, M and Trilles, J.-P. (1991).** $Na^{+}$-$K^{+}$ ATPase and carbonic anhydrase activities in larvae, postlarvae and adults of the shrimp *Penaeus japonicus* (Decapoda, Penaeidea). *Comp. Biochem. Physiol.* Vol. 100A(No.2): 433-437.

**Bouaricha, N., Charmantier-Daures, M., Thuet, P., Trilles, J.-P. and Charmantier, G. (1994).** Ontogeny of osmoregulatory structures in the shrimp *Penaeus japonicus* (Crustacea, Decapoda). *Biol. Bull.* 186: 29-40.

**Brito, R., Chimal M. and Rosas, C. (2000).** Effect of salinity in survival, growth, and osmotic capacity of early juveniles of *Farfantepenaeus brasilienses* (Decapoda: Penaeidae). *J. Exp. Mar. Biol. Ecol.* 244: 253-263.

**Brown, A. C. and Terwilliger, N.B. (1992).** Developmental changes in ionic and osmotic regulation in the Dungeness Crab, *Cancer magister*. *Biol. Bull.* 182: 270-277.

**Brown, A. C. and Terwilliger, N.B. (1999).** Developmental changes in oxygen uptake in *Cancer magister* (Dana) in response to changes in salinity and temperature. *J. Exp. Mar. Biol. Ecol.* 241: 179-192.

**Brown, S. D., Bert, T.M., Tweedale, W.A., Tores, J.J. and Lindberg, W.J. (1992).** The effects of temperature and salinity on survival and development of early life stage Florida stone crabs *Menippe mercenaria* (Say). *J. Exp. Mar. Biol. Ecol.* 157: 115-136.

**Burton, R. F. (1986).** Ionic regulation in Crustacea: the influence of temperature on apparent set points. *Comp. Biochem. Physiol.* Vol. 84A(No.1): 135-139.

**Cala, P. M. (1974).** Volume regulation by flounder (*Pseudopleuronectes americanus*) red blood cells in anisotonic media. Ph.D. Thesis, Case Western Reserve University, Cleveland, Ohio. (cited in Schmidt-Nielsen, 1975).

**Cantelmo, A. C. (1977).** Water permeability of isolated tissues from Decapod crustaceans - 1. Effects of osmotic conditions. *Comp. Biochem. Physiol.* 58A: 343-348.

**Castilho, P. C., Martins, I.A. and Bianchini, A. (2001).** Gill $Na^+$, $K^+$ - ATPase and osmoregulation in the estuarine crab, *Chasmagnathus granulata* Dana, 1851 (Decapoda, Grapsidae). *J. Exp. Mar. Biol. Ecol.* 256: 215-227.

**Castille Jr., F. L. and Lawrence, A.L. (1981).** The effect of salinity on the osmotic, sodium and chloride concentrations in the hemolmph of euryhaline shrimp of the genus *Penaeus*. *Comp. Biochem. Physiol.* Vol. 68A: 75-80.

**Charmantier, G. and Aiken, D.E. (1987).** Osmotic regulation in late embryos and prelarvae of the American lobster *Homarus americanus* H. Milne-Edwards, 1837 (Crustacea, Decapoda). *J. Exp. Mar. Biol. Ecol.* 109: 101-108.

**Charmantier, G., Charmantier-Daures, M., Bouaricha, N., Thuet, P., Aiken, D.E. and Trilles, J.P. (1988).** Ontogeny of osmoregulation and salinity tolerance in two decapod crustaceans; *Homarus americanus* and *Penaeus japonicus. Biol. Bull.* 175: 102-110.

**Charmantier, G. and Charmantier-Daures, M. (1991).** Ontogeny of osmoregulation and salinity tolerance in *Cancer irroratus*; Elements of comparison with *C. borealis* (Crustacea, Decapoda). *Biol. Bull.* 180: 125-134.

**Charmantier, G. (1998).** Ontogeny of osmoregulation in crustaceans: a review. *Invertebrate Reproduction and Development.* 33(2-3): 177-190.

**Charmantier, G., Charmantier-Daures, M. and Anger K. (1998).** Ontogeny of osmoregulation in the grapsid crab *Armases miersii* (Crustacea, Decapoda). *Mar. Ecol. Prog. Ser.* 164: 285-292.

**Charmantier, G., Haond, C., Lignot, J. and Charmantier-Daures, M. (2001).** Ecophysiological adaptation to salinity throughout a life cycle: a review of homarid Lobsters. *J. Exp. Biol.* 204: 967-977.

**Charmantier, G. and Charmantier-Daures, M. (2001).** Ontogeny of osmoregulation in crustaceans: the embryonic phase. *Am. Zool.* 41: 1078-1089.

**Charmantier, G., Gimenex, L., Charmantier-Daures, M. and Anger, K. (2002).** Ontogeny of osmoregulation, physiological plasticity and larval export strategy in the grapsid crab *Chasmagnathus granulata* (Crustacea, Decapoda). *Mar. Ecol. Prog. Ser.* 229: 185-194.

**Chen, J., and Lin, J. (1994).** Responses of osmotic and chloride concentrations of *Penaeus chinensis* Osbeck subadults acclimated to different salinity and temperature levels. *J. Exp. Mar. Bio. Ecol.* 179: 267-278.

**Chen, J., and Chia, P. (1996).** Oxygen uptake and nitrogen excretion of juvenile *Scylla serrata* at different temperature and salinity levels. *J. Crust. Biol.* 16(3): 437-442.

**Cheung, T. S. (1966).** The development of egg-membranes and egg attachment in the shore crab, *Carcinus maenas*, and some related decapods. *J. Mar. Biol. Ass. U.K.* 46: 373-400.

**Cioffi, M. (1984).** Comparative ultrastructure of arthropod transporting epithelia. *Amer. Zool.* 24: 139-156.

**Clark, R.L. (1987).** Aspects of growth and reproduction of the hairy-handed crab, *Hemigrapsus crenulatus* (Brachyura: Grapsidae). M.Sc. Thesis, University of Canterbury, 159p.

**Clarke, A. (1993).** Egg size and egg composition in polar shrimps (Caridea; Decapoda). *J. Exp. Mar. Bio. Ecol.* 168: 189-203.

**Compére, P., Wanson, S., Pequeux, A., Gilles, R. and Goffinet, G. (1989).** Ultrastructural changes in the gill epithelium of the green crab Carcinus maenas in relation to external salinity. *Tissue & Cell* 21: 299-318.

**Conte, F. P., Hootman, S.R. and Harris, P.J. (1972).** Neck organ of *Artemia salina* Nauplii. *J. Comp. Physiol* 80: 239-246.

**Corotto, F. S. and Holliday, C.W. (1996).** Branchial Na, K-ATPase and osmoregulation in the Purple Shore Crab, *Hemigrapsus nudus* (Dana). *Comp. Biochem. Physiol.* Vo. 113A(No. 4): 361-368.

**Costa, C. J., Pierce, S.K. and Warren, M.K. (1980).** The intracellular mechanism of salinity tolerance in Polychaetes: volume regulation by isolated *Glycera dibranchiata* red coelomocytes. *Biol. Bull.* 159: 626-638.

**Costello, D. P. (1939).** The volumes occupied by the formed cytoplasmic components in marine eggs. XII(1): 13-22.

**Costlow, J. D., Bookhout, C.G. and Monroe, R.J. (1966).** Studies on the larval development of the crab, *Rhithropanopeus harrisii* (Gould) I. The effect of salinity and temperature on larval development. *Physiol. Zool.* XXXIX(2): 81-100.

**Croghan, P. C. (1958a).** The osmotic and ionic regulation of *Artemia salina* (L.). *J. Exp. Biol.* 35: 219-233.

**Dalla Via, G. (1987).** Effects of salinity and temperature on oxygen consumption in a freshwater population of *Palaemonetes antennarius* (Crustacea, Decapoda). *Comp. Biochem. Physiol.* 88A(No.2): 299-305.

**Davis, C. C. (1959).** Osmotic hatching in the eggs of some fresh-water copepods. *Biol. Bull.* 116: 15-29.

**Davis, C. C. (1981).** Mechanisms of hatching in aquatic invertebrate eggs. II. *Oceanogr. Mar. Biol. Ann.* Rev. 19: 95-123.

**Dawirs, R. R. (1983).** Respiration, energy balance and development during growth and starvation of *Carcinus maenas* L. larvae (Decapoda: Portunidae). *J. Exp. Mar. Bio. Ecol.* 69: 105-128.

**De Vries, M. C. and Forward, R.B., Jr. (1991).** Mechanisms of crustacean egg hatching: evidence for enzyme release by crab embryos. *Mar. Biol.* 110: 281-291.

**Dehnel, P. A. and Carefoot, T.H. (1965).** Ion regulation in two species of intertidal crabs. *Comp. Biochem. Physiol.* Vol. 15: 377-397.

**Dehnel, P. A. (1967).** Osmotic and ionic regulation in estuarine crabs. *Estuaries: (epe):* 541-547.

**Dickson, J. S. and Dillaman, R.M. (1991).** Distribution and characterization of ion transporting and respiratory filaments in the gills of *Procambarus clarkii. Biol. Bull.* 180: 154-166.

**Diesel, R. S., M. (1998).** Effects of salinity and starvation on larval development of the crabs *Armases ricordi* and *A. roberti* (Decapoda: Grapsidae) from Jamaica, with notes on the biology and ecology of adults. *J. Crust. Biol.* 18(3): 423-436.

**D'orazio, S. E. and Holliday, C. W. (1985).** Gill Na,K-ATPase and osmoregulation in the sand fiddler crab, *Uca pugilator. Physiol. Zool.* 58(4): 364-373.

**Einarson, S. (1993).** Effect of temperature, seawater osmolality and season on oxygen consumption and osmoregulation of the amphipod *Gammarus oceanicus. Mar. Biol.* 117: 599-606.

**El-Sherief, S. S. (1990).** SEM study on the structural properties and the site of formation of egg membranes of *Carcinus maenas* (L*). Ind. J. Z. Spect. 1*(No. 2 (1-9)): 1-13.

**El-Sherief, S. S. (1993).** Histochemical characteristics of the egg membranes of *Portunus pelagicus* (L.). *Acta Biologica Hungarica.* 44(2-3): 269-280.

**Encyclopaedia Britannica (1979).** The New Encyclopaedia Britannica, 15th edition. Encyclopaedia Britannica Inc. William Benton Publisher, Chicago.

**Engelhardt, F. R. and Dehnel, P.A. (1973).** Ionic regulation in the pacific edible crab, *Cancer magister* (Dana). *Can. J. Zool.* 51: 735-743.

**Epel, D. (1972).** Activation of $Na^+$-dependent amino acid transport system upon fertilization of sea urchin eggs. *Experimental Cell Research.* 72: 74-89.

**Ewing, R. D., Peterson, G.L. and Conte F.P. (1974).** Larval salt gland of *Artemia salina* nauplii. *J. Comp. Physiol.* 88: 217-234.

**Felder, J. M., Felder D.L. and Hand, S.C. (1986).** Ontogeny of osmoregulation in the estuarine ghost shrimp *Callianassa jamaicense* var. *louisianensis* Schmitt (Decapoda, Thalassinidea). *J. Exp. Mar. Biol. Ecol.* 99: 91-105.

**Fernandez, M., Bock, C. and Portner, H. (2000).** The cost of being a caring mother: the ignored factor in the reproduction of marine invertebrates. *Ecology Letters* 3: 487-494.

**Ferrais, R. P., Parado-Estepa, F.D., Jesus, E.G. de and Ladja, J.M. (1987).** Osmotic and chloride regulation in the hemolymph of the tiger prawn *Penaeus monodon* during molting in various salinities. *Mar. Biol.* 95: 377-385.

**Fioroni, V. P. (1980).** The dorsal organ of arthropods with special reference to crustacea malacostraca- a comparative embryological survey. *Zool. J. Anat.* 104: 425-465.

**Flik, G. and Haond, C. (2000).** $Na^+$ and $Ca^{2+}$ pumps in the gills, epipodites and brachiostegites of the european lobster *Homarus gammarus*: effects of diluted sea water. *J. Exp. Biol.* 203: 213-220.

**Foskett, J. K. (1977).** Osmoregulation in the larvae and adults of the grapsid crab *Sesarma reticulatum* Say. *Biol. Bull.* 153: 505-526.

**Furspan, P., Prange, H.D. and Greenwald, L. (1984).** Energetics and osmoregulation in the catfish, I*ctalurus nebulosus* and *I. punctatus*. *Comp. Biochem. Physiol.* 77A(No. 4): 773-778.

**Gaudy, R. and Sloane, L. (1981).** Effect of salinity on oxygen consumption in postlarvae of the penaeid shrimps *Penaeus monodon* and *P. stylirostris* without and with acclimation. *Mar. Biol.* 65: 297-301.

**Geddes, M. C. (1975).** Studies on an Australian brine shrimp, *Parartemia zietziana* Sayce (Crustacea : Anostraca) - II. Osmotic and Ionic regulation. *Comp. Biochem. Physiol.* Vol. 51A: 561-571.

**Gerard, J.F. and Gilles, R. (1972).** The free amino-acid pool in *Callinectes sapidus* (Rathbun) tissues and its role in the osmotic intracellular regulation. *J. Exp. Mar. Biol. Ecol.* 10:125-136.

**Gilles, R. (1977).** Effects of osmotic stresses on the proteins concentration and pattern of *Eriocheir sinensis* blood. *Comp. Biochem. Physiol.* 56A: 109-114.

**Gilles, R. (1979).** Intracellular osmotic effectors. In: Gilles, R. (ed.), *Mechanisms of osmoregulation in animals: maintenance of cell volume*, Wiley, Chichester, pp 111-154.

**Gilles, R. (1983).** Volume maintenance and regulation in animal cells: some features and trends. *Mol. Physiol.* 4:3-16.

**Gimenez, L. and Anger, K. (2001).** Relationships among salinity, egg size, embryonic development, and larval biomass in the estuarine crab *Chasmagnathus granulata* Dana, 1851. *J. Crust. Biol.* 260: 241-257.

**Gordon, M. S. (1977).** *Animal physiology: principles and adaptations*, Chapter 7: Water and solute metabolism. New York, Macmillan Publishing Co., Inc.

**Goudeau, M. and F. Lachaise (1983).** Structure of the egg funiculus and deposition of embryonic envelopes in a crab. *Tissue & Cell.* 15: 47-62.

**Gross, W. J. (1958).** Potassium and Sodium regulation in an intertidal crab. *Biol. Bull.* 114: 334-347.

**Guerin, J. L. and Stickle, W.B. (1997).** Effect of salinity on survival and bioenergetics of juvenile lesser blue crabs, *Callinectes similis. Mar. Biol.* 129: 63-69.

**Hagedorn, M., Kleinhans F.W., Freitas, R., Liu, J. Hsu, E.W., Wildt, D.W. and Rall, W.F. (1997).** Water distribution and permeability of zebrafish embryos, *Brachydanio rerio. J. Exp. Zool.* 278: 356-371.

**Haney, D. C. and Nordlie, F.G. (1997).** Influence of Environmental Salinity on Routine Metabolic Rate and Critical Oxygen Tension of *Cyprinodon variegatus. Physiol. Zool.* 70(5): 511-518.

**Hannan, J. V. and Evans, D. H. (1973).** Water permeability in some euryhaline decapods and *Limulus polyphemus. Comp. Biochem. Physiol.* Vol. 44A: 1199-1213.

**Haond, C., Bonnal, L., Sandeaux, R., Charmantier, G. and Trilles, J.P. (1999).** Ontogeny of intracellular isosmotic regulation in the European Lobster *Homarus gammarus* (L.). *Physiol. Biochem. Zool.* 72(5): 534-544.

**Harris, R. R. and Santos, M.C.F. (1993).** Sodium uptake and transport ($Na^+ + K^+$) ATPase changes following $Na^+$ depletion and low salinity acclimation in the Mangrove crab *Ucides cordatus* (L.). *Comp. Biochem. Physiol.* 105A: 35-42.

**Harris, R. R. and D. Bayliss (1988).** Gill ($Na^+ + K^+$)-ATPases in Decapod Crustaceans: Distribution and characteristics in relation to $Na^+$ regulation. *Comp. Biochem. Physiol.* Vol. 90A(No. 2): 303-308.

**Hartnoll, R. G. and Paul, R.G.K. (1982).** The embryonic development of attached and isolated eggs of *Carcinus maenas. International Journal of Invertebrate Reproduction.* 5: 247-252.

**Hason, N., Eddy, F.B. and Flik, G. (1997).** *Ion regulation in animals.* Berlin, Germany, Springer-Verlag.

**Hayes, F. R., Darcy, D.A., and Sullivan, C.M. (1946).** Changes in the inorganic constituents of developing Salmon eggs. *The Journal of Biological Chemistry.* 163: 621-631.

**Helluy, S. M. and Beltz, B.S. (1991).** Embryonic development of the american lobster (*Homarus americanus*): quantitative staging and characterization of an embryonic molt cycle. *Biol. Bull.* 180: 355-371.

**Hicks, G. R. F. (1973).** Combined effects of temperature and salinity on *Hemigrapsus edwardsi* (Hilgendorf) and *H. crenulatus* (Milne Edwards) from Wellingon Harbour, New Zealand. *J. Exp. Mar. Bio. Ecol.* Vol. 13: 1-14.

**Holliday, C. W. (1985).** Salinity-induced changes in gill $Na^+$,$K^+$-ATPase activity in the Mud Fiddler Crab, *Uca pugnax*. *J. Exp. Zool.* 233: 199-208.

**Holliday, C. W. (1988).** Branchial $Na^+/K^+$ -ATPase and osmoregulation in the Isopod, *Idotea wosnesenskii*. *J. Exp. Biol.* 136: 259-272.

**Holliday, C. W., Roye, D.B. and Roer, R.D. (1990).** Salinity-induced changes in branchial $Na^+/K^+$ - ATPase activity and transepithelial potential difference in the Brine Shrimp *Artemia salina*. *J. Exp. Biol.* 151: 279-296.

**Holliday, C. W. and Roye D.B. (1990).** Salinity-induced changes in branchial $Na^+/K^+$ - ATPase activity and transepithelial potential difference in the brine shrimp *Artemia salina*. *J. Exp. Biol.* 151: 279-296.

**Hootman, S. R., Harris, P.J. and Conte, F.P. (1972).** Surface specialization of the larval salt gland in *Artemia salina* Nauplii. *J. Comp. Physiol.* 79: 97-104.

**Hootman, S. R. and Conte, F.P. (1975).** Functional morphology of the neck organ in *Artemia salina* Nauplii. *Journal of Morphology*. 145: 371-386.

**Howe, N. R., Quast, W.D. and Cooper, L.M. (1982).** Lethal and sublethal effects of a stimulated salt brine effluent on adults and subadults of the shrimps *Penaeus setiferus* and *P. aztecus*. *Mar. Biol.* 68: 37-47.

**Hukuda, K. (1932).** Change of weight of marine animals in diluted media. *J. Exp. Biol.* 9: 61-68.

**Hunter, K. C. and Rudy Jr., P.P. (1975).** Osmotic and ionic regulation in the Dungeness crab, *Cancer magister* Dana. *Comp. Biochem. Physiol.* Vol. 51A: 439-447.

**Hwang, P., Lee, T., Weng, C., Fang, M. and Cho, G. (1999).** Presence of Na-K-ATPase in mitochondria-rich cells in the yolk-sac epithelium of larvae of the teleost *Oreochromis mossambicus*. *Physiol. Biochem. Zool.* 72(2): 138-144.

**Jackson, M. M. (1976).** Osmotic and ionic regulation in *Hemigrapsus crenulatus* (Milne-Edwards). M.Sc Thesis, University of Canterbury: 1-62.

**Jensen, A. M. (1987).** Water balance in developing eggs of the cod *Gadus morhua*. *Fish Physiology and Biochemistry*. 3(1): 17-24.

**Jensen, A. M., Waiwood, K.G., and Peterson, R.H. (1993).** Water balance in eggs of striped bass *Morone saxatilis*. *J. Fish Biol.* 43: 345-353.

**Jensen, M. K. and Madsen, S.S. (1998).** Osmoregulation and salinity effects on the expression and activity of $Na^+$, $K^+$-ATPase in the gills of European Sea Bass, *Dicentrarchus labrax* (L.). *J. Exp. Zool.* 282: 290-300.

**Jones, M. B. (1976).** Limiting factors in the distribution of intertidal crabs (Crustacea: Decapoda) in the Avon-Heathcote estuary, Christchurch. *N.Z. J. Mar. Freshwat. Res.* 10(4): 577-587.

**Jones, M. B. and Simons, M.J. (1982).** Habitat preferences of two estuarine burrowing crabs *Helice crassa* Dana (Grapsidae) and *Macrophthalmus hirtipes* (Jacquinot) (Ocypodidae). *J. Exp. Mar. Biol. Ecol.* 56: 49-62.

**Jones, M. B. and Simons, M.J. (1982).** Response of embryonic stages of the estuarine mud crab, *Macrophthalmus hirtipes* (Jacquinot), to salinity. *International Journal of Invertebrate Reproduction.* 4: 273-279.

**Jurss, K., Bittorf, T.H. and Vokler, T.H. (1985).** Influence of salinity and ratio of lipid to protein in diets on certain enzyme activities in rainbow trout (*Salmo gairdneri* Richardson). *Comp. Biochem. Physiol.* Vol.81B(No.1): 73-79.

**Jurss, K., Bittorf, T.H., Vokler, T.H. and Wacke, R. (1983).** Influence of nutrition on biochemical sea water adaptation of the rainbow trout (*Salmo gairdneri* Richardson). *Comp. Biochem. Physiol.* Vol.75B (No. 4): 713-717.

**Jury, S. H., Kinnison, M.T., Howell, W.H. and Watson, W.H. (1994).** The effects of reduced salinity on lobster (*Homarus amerianus* Milne-Edwards) metabolism: implications for estuarine populations. *J. Exp. Mar. Biol. Ecol.* 176: 167-185.

**Kalber, F. A. and Costlow, J.D. (1966).** The ontogeny of osmoregulation and its neurosecretory control in the decapod crustacean, *Rhithropanopeus harrisii* (Gould). *Am. Zool.* 6: 221-229.

**Kalber, F. A. and Costlow, J.D. (1968).** Osmoregulation in larvae of the land-crab, *Cardisoma guanhumi* Latreille. *Am. Zool.* 8: 411-416.

**Kalman, S. M. (1959).** Sodium and water exchange in the trout egg. *J. Cell. Comp. Physiol.* 54: 155-162.

**Kao, C. Y. (1956).** Pressure-volume relationship of the *Fundulus* egg in sea water and in sucrose. *J. Gen. Physiol.* 40(1): 91-105.

**Kelly, S. P., Chow, N.K. and Woo, N.Y.S. (1999).** Alterations in $Na^+$ - $K^+$ - ATPase activity and gill chloride cell morphometrics of juvenile black sea bream (*Mylio macrocephalus*) in response to salinity and ration size. *Aquaculture.* 172: 351-367.

**Kevers, C., Pequeux, A. and Gilles, R. (1979).** Effects of an hypo-osmotic shock on $Na^+$, $K^+$ and $Cl^-$ levels in isolated axons of *Carcinus maenas*. *J. Comp. Physiol.* 129: 365-371.

**Kikuchi, S. and Matsumasa, M. (1993).** The osmoregulatory tissue around the afferent blood vessels of the coxal gills in the estuarine amphipods, *Grandidierella japonica* and *Melita setiflagella*. *Tissue & Cell* 25(4): 627-638.

**Kikuchi, S., Matsumasa, M. and Yashima, Y. (1993).** The ultrastructure of the sternal gills forming a striking contrast with the coxal gills in a fresh-water amphipod (Crustacea). *Tissue & Cell.* 25(6): 915-928.

**Kikuchi, S., Matsumasa, M. (1997).** Ultrastructural evidence for osmoregulatory function of the sternal epithelia in some Gammaridean Amphipods. *J. Crust. Biol.* 17(3): 377-388.

**Kinne, O. (1971).** Salinity - invertebrates. In: Kinne, O. (Ed), *Marine Ecology*: A comprehensive, integrated treatise on life in oceans and coastal waters; 1. Environmental Factors: Part 2., Wiley, London, pp 821-995.

**Kirschner, L. B. (1979).** Control of the extracellular fluid osmolarity, control mechanisms in crustaceans and fishes. In: Gilles, R. (ed.) Mechanisms of osmoregulation in animals, pp 157-222, Wiley, New York.

**Koop, J. H. E. and Grieshaber, M.K. (2000).** The role of ion regulation in the control of the distribution of Gammarus tigrinus (Sexton) in salt-polluted rivers. *J. Comp. Physiol. B.* 170: 75-83.

**Krogh, A. (1939).** *Osmotic regulation in aquatic animals.* Cambridge University Press, Cambridge. Pp 242.

**Kutty, M. N., Murugapoopathy, G. and Krishnan, T.S. (1971).** Influence of salinity and temperature on the oxygen consumption in young juveniles of the Indian Prawn *Penaeus indicus. Mar. Biol.* 11: 125-131.

**Lardies, M. A. and Wehrtmann, I.S. (1996).** Aspects of the reproductive biology of *Petrolisthes laevigatus* (Guerin, 1835) (Decapoda, Anomura, Porcellanidae) Part I: Reproductive output and chemical composition of eggs during embryonic development. *Arch. Fish. Mar. Res.* 43(2): 121-135.

**Lawinski, L. and B. Weglarska (1959).** Morphological and histochemical investigations on the embryological development of the crab Rhithropanopeus harrissi (Gould) subsp. *tridentata* (Maitl.). *Acta Biologica et Medica Society Science Gedan.* 3: 1-17.

**Leader, J. P. and Bedford J.J. (1978).** Volume regulation in vitro of muscle fibres of the Crab, *Hemigrapsus edwardsi. J. Comp. Physiol.* 128: 153-159.

**Lee, K. and Watts S.A. (1990).** Potential modulation of Na,K-ATPase by amines in the brine shrimp *Artemia. Amer. Zool.* 30(62A).

**Lee, K. R. and Watts, S.A. (1994).** Specific activity of $Na^+$ $K^+$ ATPase is not altered in response to changing salinities during early development of the brine shrimp *Artemia franciscana. Physiol. Zool.* 67(4): 910-924.

**Leelapiyanart, N. (1996).** Ecophysiological studies on developing eggs and ovigerous females of intertidal crabs. Ph. D. thesis, University of Canterbury.

**Lemos, D., Phan, V.N. and Alvarez, G. (2001).** Growth, oxygen consumption, ammonia-N excretion, biochemical composition and energy conent of *Farfantepenaeus paulensis* Perex-Farfante (Crustacea, Decapoda, Penaeidae) early postlarvae in different salinities. *J. Exp. Mar. Biol. Ecol.* 261: 55-74.

**Leong, P. K. K. and Manahan, D.T. (1997).** Metabolic importance of $Na^+/K^+$ -ATPase activity during sea urchin development. *J. Exp. Biol.* 200: 2881-2892.

**Lima, A. G., McNamara, J.C. and Terra, W.R. (1997).** Regulation of hemolymph osmolytes and gill $Na^+$ / $K^+$ - ATPase activities during acclimation to saline media in the freshwater shrimp *Macrobrachium olfersii* (Wiegmann, 1836) (Decapoda, Palaemonidae). *J. Exp. Mar. Biol. Ecol.* 215: 81-91.

**Lindhjem, P., Knott, B., Griffin, B. & Withers, P. (2000).** Four functional filaments of the freshwater crayfish *Cherax tenuimanus* (Decapoda: Parastacidae). *J. Crust. Biol.* 20(3): 442-452.

**Lockwood, A. P. M. and Inman C.B.E. (1973).** Changes in the apparent permeability to water at moult in the amphipod *Gammarus duebeni* and the Isopod *Idotea linearis*. *Comp. Biochem. Physiol.* Vol. 44A: 943-952.

**Lockwood, P. M. and Andrews W.R.H. (1969).** Active transport and sodium fluxes at moult in the amphipod, *Gammarus duebeni*. *J. Exp. Biol.* 51: 591-605.

**Lowy, R. J. (1984).** Isolation of salt glands from the larval brine shrimp *Artemia salina*. *Am. Zool.* 24: 265-274.

**Lowy, R. J. and Conte F.P. (1985).** Isolation and functional characterization of crustacean larval salt gland. *Am. J. Physiol.* 248: R702-R708.

**Lucu, C. (1990).** Ion regulatory mechanisms in crustacean gill epithelia. *Comp. Biochem. Physiol.* 97A(No.3): 297-306.

**Lucu, C. (1993).** Ion transport in the gill epithelium of aquatic crustacea. *J. Exp. Zool.* 265: 378-386.

**Lucu, C. and Devescovi, M. (1999).** Osmoregulation and branchial $Na^+$, $K^+$ - ATPase in the lobster *Homarus gammarus* acclimated to dilute seawater. *J. Exp. Mar. Biol. Ecol.* 234: 291-304.

**Lucu, C. and Flik, G. (1999)** $Na^+$ - $K^+$ - ATPase and $Na^+$ / $Ca^{2+}$ exchange activities in gills of hyperegulating *Carcinus maenas*. *The American Physiological Society.* R490-R499.

**Lucu, C. and Siebers D. (1987).** Linkage of $Cl^-$ fluxes with ouabain sensitive Na/K exchange through *Carcinus* gill epithelia. *Comp. Biochem. Physiol.* Vol. 87A(No. 3): 807-811.

**Maloiy, G. M. O. (1979).** *Comparative physiology of osmoregulation in Animals*, Volume 1, Chapter 2A. London, Academic Press.

**Mangum, C. and Winkle, W.V. (1973).** Responses of aquatic invertebrates to declining oxygen conditions. *Am. Zool.* 13: 529-541.

**Mani-Ponset, L., Guyot, E., Diaz, J. P. and Connes, R. (1996).** Utilization of yolk reserve during post-embryonic development in three teleostean species: the sea bream

*Sparus aurata*, the sea bass *Dicentrarchus labrax*, and the pike-perch *Stizostedion lucioperca*. *Mar. Biol.* 126: 539-547.

**Mantel, L. H. and Farmer, L. L. (1983).** Osmotic and ionic regulation. *In The Biology of Crustacea, Internal Anatomy and Physiological Regulation.* New York, Academic Press. Vol. 5, 53-161.

**Martin, J. W., Laverack, M.S. (1992).** On the distribution of the crustacean dorsal organ. *Acta Zoologica* (Stockholm) 73(5): 357-368.

**McAllen, R. and Taylor, A. (2001).** The effect of salinity change on the oxygen consumption and swimming activity of the high-shore rockpool copepod *Tigriopus brevicornis*. *J. Exp. Mar. Biol. Ecol.* 263: 227-240.

**McLay, C. L. (1988).** Brachyura and crab-like Anomura of New Zealand, Leigh Laboratory Bulletin No. 22. 280-293.

**Meschenmoser, M. (1989).** Ultrastructure of the embryonic dorsal organ of *Orchestia cavimana* (Crustacea, Amphipoda); with a note on localization of chloride and on the change in calcium-deposition before the embryonic moult. *Tissue & Cell.* 21(3): 431-442.

**Mitsunnaga-Nakatsubo, K., Fujiwara, A. and Yasumasu, I. (1992).** Change in the activity of $Na^+$, $K^+$-ATPase in embryos of the sea urchin, *Hemicentrotus pulcherrimus*, during early development. *Dev. Growth Differ.* 34: 379-385.

**Modlin, R. F., Froelich, A.J. (1997).** Influence of temperature, salinity, and weight on the oxygen consumption of a laboratory population of *Americamysis bahia* (Mysidacea). *J. Crust. Biol.* 17(1): 21-26.

**Moloo, S. K. (1971).** Some aspects of water absorption by the developing egg of *Schistocerca gregaria*. *J. Insect Physiol.* 17: 1489-1495.

**Moore, M. L. and Richardson, A.M.M. (1992).** Water uptake and loss via the urosome in terrestrial talitrid amphipods (Crustacea: Amphipoda). *Journal of Natural History.* 26: 67-77.

**Morgan, J. D. and Iwama, G.K. (1998).** Salinity effects on oxygen consumption, gill $Na^+$, $K^+$ -ATPase and ion regulation in juvenile coho salmon. *J. Fish Biol.* 53: 1110-1119.

**Morris, S. and Edwards T. (1995).** Control of osmoregulation via regulation of $Na^+/K^+$ - ATPase activity in the amphibious purple shore crab *Leptograpsus variegatus*. *Comp. Biochem. Physiol.* 112C: 129-136.

**Morritt, D. and Spicer, J. I. (1995).** Changes in the pattern of osmoregulation in the brackish water amphipod *Gammarus duebeni* Lilljeborg (Crustacea) during embryonic development. *J. Exp. Zool.* 273: 271-281.

**Morritt, D. and Spicer, J. I. (1996).** Developmental ecophysiology of the beachflea *Orchestia gammarellus* (Pallas) (Crustacea: Amphipoda) II. Embryonic osmoregulation. *J. Exp. Mar. Biol. Ecol.* 207: 205-216.

**Morton, J. and Miller, M. (1968).** The New Zealand sea shore. Collins, Auckland, 638p. (cited in McLay, 1988).

**Naylor, J. K., Taylor, E.W. and Bennett, D.B. (1997).** The oxygen uptake of ovigerous edible crabs (*Cancer pagurus*) (L.) and their eggs. *Mar. Fresh. Behav. Physiol.* 30: 29-44.

**Naylor, J. K., Taylor, E.W. and Bennett, D.B. (1999).** Oxygen uptake of developing eggs of *Cancer pagurus* (Crustacea: Decapoda: Cancredae) and consequent behaviour of the ovigerous females. *J. Mar. Biol. Ass. U.K.* 79: 305-315.

**Needham, J. (1933).** The energy sources in ontogenesis, VII. The respiratory quotient of developing crustacean embryos. *J. Exp. Biol.* 10: 79-87.

**Nye, P. A. (1977).** Reproduction, growth and distribution of the grapsid crab *Helice crassa* (Dana, 1851) in the southern part of New Zealand. *Crustaceana.* 33(1): 75-89.

**Pandian, T. J. (1970).** Ecophysiological studies on the developing eggs and embryos of the European lobster *Homarus gammarus*. *Mar. Biol.* 5: 154-167.

**Pandian, T. J. (1970).** Yolk utilization and hatching time in the canadian lobster *Homarus americanus*. *Mar. Biol.* 7: 249-254.

**Pequeux, A. (1995).** Osmotic regulation in crustaceans. *J. Crust. Biol.* **15**(1): 1-60.

**Pequeux, A. J. R. and Gilles, R. (1984).** *Control of the extracellular fluid osmolality in crustaceans*. Berlin, Germany, Springer-Verlag.

**Perkins, H. C. (1972).** Developmental rates at various temperature of embryos of the northern lobster (*Homarus americanus* Milne – Edwards). *Fish. Bull.* 70(1):95-99.

**Perry, S. F. and McDonald, G. (1993).** Gas exchange. *The Physiology of fishes. Ed. Evans, D.H.* Fla, CRC, Boca Raton. 251-278

**Peterson, G. L. (1978).** A simplified method for analysis of inorganic phosphate in the presence of interfering substances. *Analytical Biochemistry.* 84: 164-172.

**Peterson, G. L., Ewing, R. D. and Conte, F.P. (1978).** Membrane differentiation and de novo synthesis of the $Na^+$-$K^+$ activated adenosine triphosphatase during development of the *Artemia salina* nauplii. *Dev. Biol.* 67: 90-98. (cited in Bouaricha, et. al., 1991)

**Peterson, R. H. and Martin-Robichaud, D.J. (1986).** Perivitelline and vitelline potentials in teleost eggs as influenced by ambient ionic strength, natal salinity, and electrode electrolyte; and the influence of these potentials on cadmium dynamics within the egg. *Can. J. Fish Aqua. Sci.* 43: 1445-1450.

**Phillips, J. C. (1968).** Osmotic and ionic regulation in *Hemigrapsus edwardsi*. Auckland, University of Auckland. M.Sc. Thesis (cited in McLay, 1988).

**Pierce, S. K. and Greenberg, M.J. (1972).** The nature of cellular volume regulation in marine bivalves. *J. Exp. Biol.* 57: 681-692.

**Pierce, S. K. and Greenberg, M.J. (1973).** The initiation and control of free amino acid regulation of cell volume in salinity-stressed marine bivalves. *J. Exp. Biol.* 59: 435-446.

**Pierce, S. K. and Greenberg, M.J. (1976).** Hypo-osmotic cell volume regulation in marine bivalves: effect of membrane potential change and metabolic inhibition. *Physiol. Zool.* 49: 417-424.

**Pierce, S. K. (1982).** Invertebrate cell volume control mechanisms: A coordinated use of intracellular amino acids and inorganic ions as osmotic solute. *Biol. Bull.* 163: 405-419.

**Piller, S. C., Henry, R. P., Doeller, J. E. and Kraus, D. W. (1995).** A comparison of the gill physiology of two euryhaline crab species, *Callinectes sapidus* and *Callinectes similis*: Energy production, transport-related enzymes and osmoregulation as a function of acclimation salinity. *J. Exp. Biol.* 198: 349-358.

**Postel, U., Petraush, G., Riestenpatt, S., Weihrauch, D., Malykh, J., Becker, W., and Siebers, D. (1998).** Inhibition of $Na^+/K^+$ - ATPase and of active ion-transport functions in the gills of the shore crab *Carcinus maenas* induced by cadmium. *Mar. Biol.* 130: 407-416.

**Potts, W. T. W. (1954).** The energetics of osmotic regulation in brackish- and fresh-water animals. *J. Exp. Biol.* 31: 618-630.

**Potts, W. T. W. and Parry, G. (1963).** *Osmotic and Ionic regulation in animals.* Oxford, Pergamon Press.

**Potts, W. T. W. and Eddy, F.B. (1973).** The permeability to water of the eggs of certain marine teleosts. *J. Comp. Physiol.* 82: 305-315.

**Potts, W. T. W. and Eddy F.B. (1973).** The permeability to water of the eggs of certain marine teleosts. *J. Comp. Physiol.* 82: 305-315.

**Potts, W. T. W. and Rudy P.P. (1969).** Water balance in the eggs of the atlantic salmon *Salmo salar*. *J. Exp. Biol.* 50: 223-237.

**Prosser, C. L. (1973).** Water: osmotic balance, hormonal regulation; inorganic ions. *In Comparative Animal Physiology.* Philadelphia, Saunders. 1-110.

**Quetin, L. B. and Ross, R.M. (1989).** Effects of oxygen, temperature and age on the metabolic rate of the embryos and early larval stages of the Antarctic krill *Euphausia superba* Dana. *J. Exp. Mar. Bio. Ecol.* 125: 43-62.

**Rankin, J. C. and Davenport J. (1981).** *Animal Osmoregulation.* Glasgow and London, Blackie & Sons Limited. 1-197.

**Rao, G. M. M. (1968).** Oxygen consumption of rainbow trout (*Salmo gairdneri*) in relation to activity and salinity. *Can. J. Zool.* 46: 781-786.

**Richmond, C. E. and Woodin, S.A. (1999).** Effect of salinity reduction on oxygen consumption by larval estuarine invertebrates. *Mar. Biol.* 134: 259-267.

**Robertson, J. D. (1970).** Osmotic and Ionic regulation in the Horseshoe crab *Limulus polyphemus* (Linnaeus). *Biol. Bull.* 138: 157-183.

**Rosas, C., Martinex, E., Gaxiola, G., Brito, R., Sanchez, A. and Soto, L.U. (1999).** The effect of dissolved oxygen and salinity on oxygen consumption, ammonia excretion and osmotic pressure of *Penaeus setiferus* (Linnaeus) juveniles. *J. Exp. Mar. Biol. Ecol.* 234: 41-57.

**Rosas, C., Ocampo, L., Gaxiola, G., Sanchez, A. and Soto, L.A. (1999).** Effect of salinity on survival, growth, and oxygen consumption of postlarvae (PL10-PL12) of *Litopenaeus setiferus. J. Crust. Biol.* 19(2): 244-251.

**Rudy, P. P. and Potts, T.W. (1969).** Sodium balance in the eggs of the Atlantic Salmon, *Salmo salar. J. Exp. Biol.* 50: 239-246.

**Saigusa, M. (1992).** Control of hatching in an estuarine terrestrial crab 1. hatching of embryos detached from the female and emergence of mature larvae. *Biol. Bull.* 183: 401-408.

**Saigusa, M. (1992).** Observations on egg hatching in the estuarine crab *Sesarma haematocheir. Pacific Science.* Vol. 46(no. 4): 484-494.

**Savage, J. P. and Robinson G.D. (1983).** Inducement of increased gill $Na^+$-$K^+$ ATPase activity by a hemolymph factor in hyperosmoregulating *Callinectes sapidus. Comp. Biochem. Physiol.* 75A(No. 1):65-69.

**Schatzlein, F. C. and Costlow, D. JR (1978).** Oxygen consumption of the larvae of the decapod crustaceans, *Emerita talpoida* (SAY) and *Liminia emarginata* LEACH. *Comp. Biochem. Physiol.* 61A:441-450.

**Schmidt-Nielsen (1983).** *Animal Physiology: Adaptation and environment*, Part Four : water. Third edition. Cambridge, Cambridge University Press. 309-360.

**Schmidt-Nielsen, B. (1975).** Comparative physiology of cellular ion and volume regulation. *J. Exp. Zool.* 194: 207-220.

**Schoffeniels, E. (1973).** *Amino acid metabolism and cell volume regulation.* Comparative Physiology, eds., L. Bolis, K. Schmidt-Nielsen & S.H.P. Maddrell. North-Holland, North-Holland Publishing Company. 354-385.

**Schuh, M. and Diesel, R. (1995).** Effects of salinity and starvation on the larval development of *Sesarma curacaoense* De Man 1892, A mangrove crab with abbreviated development (Decapoda: Grapsidae). *J. Crust. Biol.* 15(4): 645-654.

**Shakuntala, K. and Reddy, S.R. (1982).** Crustacean egg size as an indicator of egg fat/protein reserves. *International Journal of Invertebrate Reproduction.* 4: 381-384.

**Shaw, J. (1955).** The permeability and structure of the cuticle of the aquatic larva of *Sialis lutaria*. *J. Exp. Biol.* 32(No. 2): 330-352.

**Shen, A. C. Y. and Leatherland, J.F. (1978).** Effect of ambient salinity on ionic and osmotic regulation of eggs, larvae, and alevins of rainbow trout (*Salmo gairdneri*). *Can. J. Zool.* 56(4): 571-577.

**Shephard, K. L. (1987).** Ion-exchange phenomena regulate the environment of embryos in the eggs of freshwater fish. *Comp. Biochem. Physiol.* 88A(4): 659-662.

**Shephard, K. L. and McWilliams, M. (1989).** Ionic regulation by the eggs of salmon. *J. Comp. Biochem. Physiol.* 159: 249-254.

**Siebers, D., Winkler, A., Lucu, C., Thedens, G., and Weichart, D. (1985).** Na-K-ATPase generates an active transport potential in the gills of the hyperregulating shore crab *Carcinus maenas*. *Mar. Biol.* 87: 185-192.

**Siebers, D., Leweck K., Markus, H. & Winkler, A. (1982).** Sodium regulation in the Shore Crab *Carcinus maenas* as related to ambient Salinity. *Mar. Biol.* 69: 37-43.

**Simons, M. J. and Jones, M.B. (1981).** Population and reproductive biology of the mud crab, *Macropthalmus hirtipes* (Jacquinot, 1853) (Ocypodidae), from marine and estuarine habitats. *Journal of Natural History.* 15: 981-994.

**Smaldon, G. (1973).** Free pool amino acids in the developing embryos of *Pisidia longicornis* (L.). (Deapoda, *Anomura*). *Comp. Biochem. Physiol.* 44B:711-714.

**Smith, R. I. (1967).** Osmotic regulation and adaptive reduction of water-permeability in a brackish-water crab, *Rhithropanopeus harrisi* (Brachyura, Xanthidae). *Biol. Bull.* 133: 643-658.

**Smith, R. I. (1969).** The ionic relations of *Artemia salina* (L.) I. Measurements of electrical potential difference and resistance. *J. Exp. Biol.* 51: 727-738.

**Smith, R. I. (1969).** The ionic relations of *Artemia salina* (L.) II. Fluxes of sodium, chloride and water. *J. Exp. Biol.* (51): 739-757.

**Smith, R. I. (1970).** The apparent water-permeability of *Carcinus maenas* (Crustacea, Brachyura, Portunidae) as a function of salinity. *Biol. Bull.* 139: 351-362.

**Smith, R. I. and Rudy P.P. (1972).** Water-exchange in the crab *Hemigrapsus nudus* measured by use of Deuterium and tritium oxides as tracers. *Biol. Bull.* 143(1): 234-246.

**Spaargaren, D. H. (1976).** A comparative study on the regulation of osmotic, ionic and organic-solute concentrations in the blood of aquatic organisms. *Comp. Biochem. Physiol.* 53A: 31-40.

**Steele, D. H. and Steele, V.J. (1975).** Egg size and duration of embryonic development in Crustacea. *Int. Revue ges. Hydrobiol.* 60(5): 711-715.

**Stein, W. D. (1967).** *The Movement of molecules across cell membranes.* London, Academic Press. Pp 361.

**Stromberg, J. (1972).** *Cyathura polita* (Crustacea, Isopoda), some embryological notes. *Bull. Mar. Sci.* 22: 463-483.

**Subramoniam, T. (1979).** Some aspects of reproductive ecology of a Mole Crab *Emerita asiatica* Milne Edwards. *J. Exp. Mar. Biol. Ecol.* Vol. 36: 259-268.

**Surbida, K.L. and Wright, J.C. (2001).** Embryo tolerance and maternal control of marsupial environment in *Armadillidium vulgare* (Isopoda:Oniscidea). *Physiol. Biochem. Zool.* 74(6): 894-906.

**Susanto, G. N. and Peterson, M.S. (1996).** Survival, osmoregulation and oxygen consumption of YOY coastal largemouth bass, *Micropterus salmoides* (Lacepede) exposed to saline media. *Hydrobiologia* 323: 119-127.

**Susanto, G. N. and Charmantier, G. (2000).** Ontogeny of osmoregulation in the crayfish *Astacus leptodactylus. Physiol. Biochem. Zool.* 73(2): 169-176.

**Susanto, G. N. and Charmantier, G. (2001).** Crayfish freshwater adaptation starts in eggs: Ontogeny of osmoregulation in embryos of *Astacus leptodactylus. J. Exp. Zool.* 289: 433-440.

**Sutcliffe, D. W. (1961).** Studies on salt and water balance in caddis larvae (Trichoptera): 1. Osmotic and ionic regulation of body fluids in *Limnephilus affinis* Curtis. *J. Exp. Biol.* 38: 501-519.

**Swanson, C. (1996).** Early development of milkfish: effects of salinity on embryonic and larval metabolism, yolk absorption and growth. *J. Fish Biol.* 48: 405-421.

**Talbot, P., Clark, W.H. and Lawrence, A.L. (1972).** Light and electron microscopic studies on osmoregulatory tissue in the developing brown shrimp, *Penaeus aztecus. Tissue & Cell.* 4(2): 271-286.

**Taylor, E. W. and Butler, P.J. (1973).** The behaviour and physiological responses of the shore crab *Carcinus maenas* during changes in environmental oxygen tension. *Netherlands Journal of Sea Research.* 7: 496-505.

**Taylor, E. W., Butler, P.J. and Al-Wassia, A. (1977).** The effect of a decrease in salinity on respiration, osmoregulation and activity in the shore crab, *Carcinus maenas* (L.) at different acclimation temperatures. *J. Comp. Physiol.* 119: 155-170.

**Taylor, H. H. (1977).** The ionic and water relations of embryos of *Lymnaea stagnalis,* a freshwater pulmonate mollusc. *J. Exp. Biol.* 69: 143-172.

**Taylor, H. H. and Leelapiyanart, N. (2001).** Oxygen uptake by embryos and ovigerous females of two intertidal crabs *Heterozius rotundifrons* (Bellidae) and *Cyclograpsus lavauxi* (Grapsidae): scaling and the metabolic costs of reproduction. *J. Exp. Biol.* 204: 1083-1097.

**Taylor, H. H. and Taylor, E. W. (1992).** Gills and lungs: the exchange of gases and ions. In Microscopic anatomy of invertebrates, Vol. 10, eds. F. W. Harrison and A. G. Humes, pp. 203-293. Wiley-Liss: New York.

**Tedengren, M., Arner, M. and Kautsky, N. (1988).** Ecophysiology and stress response of marine and brackish water *Gammarus* species (Crustacea, Amphipoda) to changes in salinity and exposure to cadmium and diesel-oil. *Mar. Ecol. Prog. Ser.* 47: 107-116.

**Terwilliger, N. B. and Brown, A.C. (1993).** Ontogeny of hemocyanin function in the Dungeness crab *Cancer magister*: The interactive effects of developmental stage and divalent cations on hemocyanin oxygenation properties. *J. Exp. Biol.* 183: 1-13.

**Thomson, G. M. and Anderton, T. (1921).** History of the Portobello marine fish-hatchery and biological station. *Dom. N.Z., Biol. Sci. Art. Bull.* 4: 131p. (cited in McLay, 1988).

**Thuet P., Charmantier-Daures M. and Charmantier, G. (1988).** Relation entre osmoregulation et activites d'ATPase $Na^+$-$K^+$ et d'anhydrase carbonique chez larves et post-larves de Homarus gammarus (L.). *J. Exp. Mar. Biol. Ecol.* 115: 249-261.

**Towle, D. W. (1984).** Membrane-bound ATPases in Arthropod Ion-Transporting tissues. *Am. Zool.* 24: 177-185.

**Towle, D. W. and Kays W.T. (1986).** Basolateral localization of $Na^+$+$K^+$-ATPase in gill epithelium of two osmoregulating crabs, *Callinectes sapidus* and *Carcinus maenas*. *J. Exp. Zool.* 239: 311-318.

**Treherne, J. E. and Pichon, Y. (1978).** Long-term adaptations of *Sabella* giant axons to hyposmotic stress. *J. Exp. Biol.* 75: 253-263.

**Valdes, L., Alvarez-Ossorio, M.T. and Gonzalez-Gurriaran, E. (1991).** Incubation of eggs of *Necora puber* (L., 1767)(Decapoda, Brachyura, Portunidae). Volume and biomass changes in embryonic development. *Crustaceana* 60(2): 163-177.

**Varsamos, G., Connes, R., Diaz, J.P., Barnable, G. (2001).** Ontogeny of osmoregulation in the European sea bass *Dicentrarchus labrax* L. *Mar. Biol.* 138: 909-915.

**Warren, M K. and Pierce, S K. (1982).** Two cell volume regulatory systems in the *Limulus myocardium:* an interaction of ions and quaternary ammonium compounds. *Biol. Bull.* 163: 504-516.

**Wear R.G. (1970).** Life-history studies on New Zealand Brachyura. 4. Zoea larvae hatched from crabs of the family grapsidae. *N.Z. J. Mar. Freshwat. Res.* 4 (1): 3 – 35. (cited in McLay, 1988).

**Wear, R. G. (1974).** Incubation in british decapod crustacea, and the effects of temperature on the rate and success of embryonic development. *J. Mar. Biol. Ass. U.K.* 54: 745-762.

**Wehrtmann, I. S. and Kattner, G. (1998).** Changes in volume, biomass, and fatty acids of developing eggs in *Nauticaris magellanica* (Decapoda, Caridea): A latitudinal comparison. *J. Crust. Biol.* 18(3): 413-422.

**Welcomme, L. and Devos, P. (1988).** Cytochrome c oxidase and $Na^+$ - $K^+$ ATPase activities in the anterior and posterior gills of the shore crab *Carcinus maenas* L. after adaptation to various salinties. *Comp. Biochem. Physiol.* 89B(No.2): 339-341.

**Wenner, E. (1992).** Introduction to session IV: mating, reproduction and development. *Am. Zool.* 32: 501-502.

**Wheatly, M. G. (1981).** The provision of oxygen to developing eggs by female shore crabs (*Carcinus maenas*). *J. Mar. Biol. Ass. U.K.* 61: 117-128.

**Wheatly, M. G. and Gannon, A.T. (1995).** Ion regulation in crayfish: freshwater adaptations and the problem of molting. *Am. Zool.* 35: 49-59.

**Williams, B. G. (1969).** The rhythmic activity of *Hemigrapsus edwardsi*. *J. Exp. Mar. Bio. Ecol.* Vol. 3: 215-223.

**Willmer, P. G. (1978).** Volume regulation and solute balance in the nervous tissue of an osmoconforming bivalve (*Mytilus edulis*). *J. Exp. Biol.* 77: 157-179.

**Winkler, A. (1986).** Effects of inorganic sea water constituents on branchial Na-K-ATPase activity in the shore crab *Carcinus maenas*. *Mar. Biol.* 92: 537-544.

**Winnicki, A. and Slomianko, M. (1970).** Taking-up of water by the eggs of the crab *Rhithropanopeus harrisi* (Gould) subsp. *tridentatus* (Maitland) during embryonic development. *Zoologica Poloniae.* 20(4): 415-422.

**Withers, P.C. (1992).** Comparative Animal Physiology. Saunders College Publishing, New York, 949 pp.

**Wolcott, D. L. (1991).** Integration of cellular, organismal, and ecological aspects of salt and water balance. *Mem. Queensland Mus.* 31: 229-239.

**Yamaguchi, T. (2001).** Incubation of eggs and embryonic development of the Fiddler Crab, *Uca lactea* (Decapoda, Brachyura, Ocypodidae). *Crustaceana.* 74(5): 449-458.

**Yancy, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D., and Somero, G. N. (1982).** Living with water stress; evolution of osmolyte systems. *Science* 217: 1214-1223.

**Yonge, C. M. (1934)** Egg attachment in *Crangon vulgaris* and other Caridea.: Proc. Zool. Soc. Lond. 107: 369-400.

**Yonge, C. M. (1937).** The Nature and significance of the membranes surrounding the developing eggs of *Homarus vulgaris* and other Decapoda. *Proc. Zool. Soc. Lon. Ser. A* 107: 499-517.

**Yonge, C. M. (1946).** Permeability and properties of the membranes surrounding the developing egg of *Homarus vulgaris*. *J. Mar. Biol. Ass. UK.* 26: 432-438.

**Zanders, I. P. (1980).** Regulation of blood ions in *Cacinus maenas* (L.). *Comp. Biochem. Physiol.* 65A:97-108.

**Zanders, I. P. and Rodrigues, J.M. (1992).** Effects of temperature and salinity stress on osmotic regulation in adults and on oxygen consumption in larvae and adults of *Macrobrachium amazonicum* (Decapoda, Palaemonidae). *Comp. Biochem. Physiol.* 101A(No. 3): 505-509.

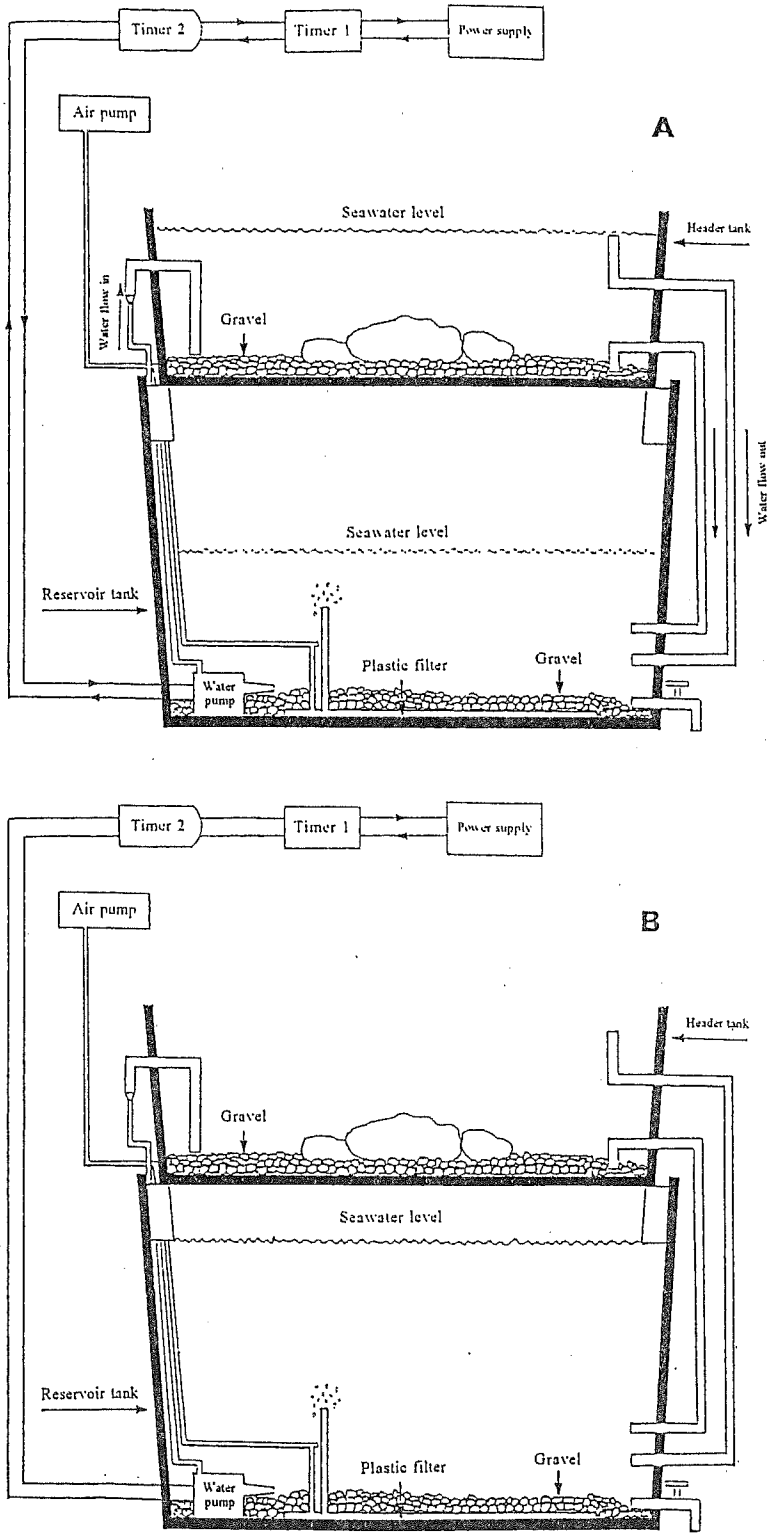
**Zare, S. and Greenaway, P. (1998).** The effect of moulting and sodium depletion on sodium transport and the activities of $Na^+$ $K^+$ -ATPase, and V - ATPase in the freshwater crayfish *Cherax destructor* (Crustacea: Parastacidae). *Comp. Biochem. Physiol.* 119A: 739-745.

# APPENDIX A

## TIDAL SYSTEM (REFERENCE ; LEELAPIYANART, 1996)

The tidal system provided alternate periods of immersion (high tide) and emersion (low tide) of equal duration (6h 12 min). Each consisted of two tanks, a filter, a small pump and two household timers. Two tanks consisted of polypropylene stacking fish boxes of similar base dimensions but of different heights. The smaller tank, 40.5 cm wide x 63.5 cm long x 21.5 cm high was used for holding crabs and rested on the larger tank (40 cm high) which was used as a reservoir. A plastic filter covered with a layer of gravel, about 5 cm thick, was placed on the bottom of the reservoir tank. An air flow system was connected to the filter and this aerated and conditioned the seawater.

High and low water was controlled by a small pump placed inside the reservoir tank. The operation of the pump was controlled by the two timers. Timer 1 (an electromechanical clock and relay) switched the pump on and off for, nominally, 6h periods. The timer 2 (an electronic clock and relay with battery backup) was set to control the tidal delay time which is 48 min per day in this tidal system (about 50 min per day on the shore naturally). This was achieved by using timer 2 to interrupt the power supply to the clock of timer 1 for 12 periods of 4 min per day. When the pump turned on, seawater in the reservoir tank was pumped into the header tank and flowed back down to the reservoir tank through two overflow pipes (the pump output exceeded the flow in the lower pipe)(Figure A). When the pump was switched off, the system drained back into the lower reservoir through the lower overflow pipe and no circulation of seawater occurred (Figure B). However, at low water, some interstitial water was retained by the gravel.




Schematic diagrams of tidal tank system (A) During high tide period (B) During low tide period.

# ACKNOWLEDGEMENTS

My most sincere thanks go to my supervisor Assoc. Prof. H.H. Taylor for his guidance and especially his patience when reading the draft chapters. His immense knowledge and understanding of my field has been invaluable to me.

My thanks are also due to Assoc. Prof. Islay Marsden and Dr. Druscilla Mason for the advice given to me during the progress report discussion times which was helpful to improve this thesis work.

My special thanks go to Jan Mckenzie for assisting me doing field collections, photography, microscopy and proof reading some of the chapters in this thesis. And also I am grateful for the expert technical guidance I received from her during the light, scanning and electron microscopic studies.

I wish to thank Gavin Robinson for all the technical support that he has given me throughout the study period and also I thank all the technical staff members, secretaries in the Dept. of Zoology who were always willing to help me in different ways.

Most importantly, thanks to all my friends, whom I have met in Christchurch who provided assistance to me in numerous ways during my study period.

This research would not have been possible without the New Zealand Overseas Development Assistance Postgraduate Scholarship and I am grateful for that.

Finally, I wish to thank my mother and sisters for giving me moral support and encouragement during the period of stay away from home to fulfil my desires. Thank you very much. I am deeply sad that my father could not be here to see me complete my thesis.